PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 48/00, C12N 5/10 — A2

(11) International Publication Number: WO 00/06204

(43) International Publication Date: 10 February 2000 (10.02.00)

(21) International Application Number: PCT/US99/16961

(22) International Filing Date: 28 July 1999 (28.07.99)

(30) Priority Data:
60/094,408 28 July 1998 (28.07.98) US

(71) Applicant: ADVANCED TISSUE SCIENCES, INC. [US/US]; 10933 North Torrey Pines Road, La Jolla, CA 92037–1005 (US).

(72) Inventors: NAUGHTON, Gail, K.; 10933 North Torrey Pines Road, La Jolla, CA 92037–1005 (US). MANSBRIDGE, Jonathon, N.; 1685 Calle Camille, La Jolla, CA 92037 (US).

(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: GENETICALLY ENGINEERED CELLS AND TISSUES

(57) Abstract

The present invention relates to cells genetically engineered to express a target gene in response to a given "environmental cue" or "stimulus", and their use for the production of three–dimensional tissues or injectable preparations which can be used in tissue repair, replacement or enhancement, and/or for the delivery of therapeutic gene products *in vivo*. In particular, the invention relates to cell and tissue bioreactors that are engineered to express a target gene product, which acts as a reporter of a chosen physiological condition, augments deficient or defective expression of a gene product or provides an anti–viral, anti–bacterial, anti–microbial, or anti–cancer activity in response to a given "environmental cue" or "stimulus".

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

GENETICALLY ENGINEERED CELLS AND TISSUES

1. INTRODUCTION

The present invention relates to cells genetically engineered to express a target gene in response to a given "environmental cue" or "stimulus", and their use in generating three-dimensional tissues having improved properties for production and processing, or superior properties for use *in vivo*, in tissue or organ repair or as bioreactors for the delivery of therapeutic products.

2. BACKGROUND OF THE INVENTION

2.1. TISSUE CULTURE SYSTEMS

The majority of vertebrate cell cultures *in vitro* are grown as monolayers on an artificial substrate bathed in nutrient medium. The nature of the substrate on which the monolayers grow may be solid, such as plastic, or semisolid gels, such as collagen or agar. Disposable plastics have become the preferred substrate used in modern-day tissue or cell culture.

A few researchers have explored the use of natural substrates related to basement membrane components for tissue culture. Basement membranes comprise a mixture of glycoproteins and proteoglycans that surround most cells *in vivo*. For example, Reid and Rojkund, 1979, in, Methods in Enzymology, Vol. 57, Cell Culture, Jakoby & Pasten, eds., New York, Acad. Press, pp. 263-278; Vlodavsky et al., 1980, Cell 19:607-617; Yang et al., 1979, Proc. Natl. Acad. Sci. USA 76:3401 have used collagen for culturing hepatocytes, epithelial cells and endothelial tissue. Growth of cells on floating collagen (Michalopoulos and Pitot, 1975, Fed. Proc. 34:826) and cellulose nitrate membranes (Savage and Bonney, 1978, Exp. Cell Res. 114:307-315) have been used in attempts to promote terminal differentiation.

Cultures of mouse embryo fibroblasts have been used to enhance the growth of cells, particularly at low densities. This effect is thought to be due partly to supplementation of the medium but may also be due to conditioning of the substrate by cell products. In these systems, feeder layers of fibroblasts are grown as confluent monolayers which make the surface suitable for the attachment of other cells. For

example, the growth of glioma cells on confluent feeder layers of normal fetal intestine has been reported (Lindsay, 1979, Nature 228:80).

While the growth of cells in two dimensions allows a high rate of cell proliferation and is a convenient method for preparing, observing and studying cells in culture, it lacks the cell-cell and cell-matrix interactions characteristic of whole tissue *in vivo*. In order to study such functional and morphological interactions, a few investigators have explored the use of three-dimensional substrates such as collagen gel (Douglas et al., 1980, In Vitro 16:306-312; Yang et al., 1979, Proc. Natl. Acad. Sci. 76:3401; Yang et al., 1980, Proc. Natl. Acad. Sci. 77:2088-2092; Yang et al., 1981, Cancer Res. 41:1021-1027); cellulose sponge, alone (Leighton et al., 1951, J. Natl. Cancer Inst. 12:545-561) or collagen coated (Leighton et al., 1968, Cancer Res. 28:286-296); and a gelatin sponge, Gelfoam (Sorour et al., 1975, J. Neurosurg. 43:742-749).

In general, these three-dimensional substrates are inoculated with the cells to be cultured. Many of the cell types have been reported to penetrate the matrix and to establish a "tissue-like" histology. For example, three dimensional collagen gels have been utilized to culture breast epithelium (Yang et al., 1981, Cancer Res. 41:1021-1027) and sympathetic neurons (Ebendal, 1976, Exp. Cell Res. 98:159-169). Additionally, various attempts have been made to regenerate tissue-like architecture from dispersed monolayer cultures. Kruse and Miedema (1965, J. Cell Biol. 27:273) reported that perfused monolayers could grow to more than ten cells deep and organoid structures can develop in multilayered cultures if kept supplied with appropriate medium (see also Schneider et al., 1963, Exp. Cell. Res. 30:449-459; Bell et al., 1979, Proc. Natl. Acad. Sci. USA 76:1274-1279; Green, 1978, Science 200:1385-1388). It has been reported that human epidermal keratinocytes may form dematoglyphs (friction ridges if kept for several weeks without transfer); Folkman and Haudenschild (1980, Nature 288:551-556) reported the formation of capillary tubules in cultures of vascular endothelial cells cultured in the presence of endothelial growth factor and medium conditioned by tumor cells; and Sirica et al. (1979, Proc. Natl. Acad. Sci. USA 76:283-287; 1980, Cancer Res. 40:3259-3267) maintained hepatocytes in primary culture for about 10-13 days on nylon meshes coated with a thin layer of collagen. However, the long term culture and proliferation of cells in such systems has not been achieved.

Indeed, the establishment of long term culture of tissues such as bone marrow has been attempted. Overall the results were disappointing, in that although a stromal cell layer containing different cell types is rapidly formed, significant hematopoiesis

could not be maintained for any real time. (For review see Dexter et al., In Long Term Bone Marrow Culture, 1984, Alan R. Liss, Inc., pp. 57-96).

A number of groups have attempted to grow skin and connective tissue *in vitro* for transplantation *in vivo*. In one such system, a hydrated bovine collagen lattice forms the substrate to which cells, such as fibroblasts are incorporated which results in the contraction of the lattice into tissue (Bell et al., U.S. Patent No. 4,485,096). In another system, a porous cross-linked collagen sponge is used to culture fibroblast cells (Eisenberg, WO 91/16010). A scaffold composed of synthetic polymers has also been described to control cell growth and proliferation *in vitro* so that once the fibroblasts begin to grow and attach to the matrix it is transplanted into the patient (Vacanti et al., U.S. Patent Nos. 5,759,830; 5,770,193; 5,736,372). Additional synthetic matrices composed of biodegradable, biocompatible copolymers of polyesters and amino acids have also been designed as scaffolding for cell growth (U.S. Patent Nos. 5,654,381; 5,709,854). Three-dimensional cell culture systems have also been designed which are composed of a stromal matrix which supports the growth of cells from any desired tissue into an adult tissue (Naughton et al., U.S. Patent Nos. 4,721,096, 4,963,489 and 5,032,508). Another approach involves slowly polymerizing hydrogels containing large numbers of the desired cell type which harden into a matrix once administered to a patient (U.S. Patent No. 5,709,854). Extracellular matrix preparations have been designed which are composed of stromal cells which provide a three dimensional cell culture system for a desired cell type which may be injected into the patient for precise placement of the biomaterial (Naughton et al., WO 96/39101).

2.2. GENE THERAPY

Gene therapy protocols are directed to the delivery of biologically active gene products or the delivery of replacement genes to correct heritable defects in target cells. There are two approaches which have been relied on to introduce genes into cells and tissues: *ex vivo* gene transfer and *in vivo* gene transfer.

Any number of transfection techniques which are used to transfer DNA *in vitro* into cells may be used for *ex vivo* gene transfer, including calcium phosphate - DNA precipitation; DEAE-Dextran transfection, electroporation, liposome mediated DNA transfer or transduction with recombinant viral vectors. These transfection techniques have been used to transfer DNA into a variety of cell types, including endothelial cells, hepatocytes, fibroblasts, lymphocytes, and hematopoietic stem cells (*e.g.*, see U.S. Patent No. 4,868,116, U.S. Patent No. 4,980,286; Morgan & Mulligan WO 87/00201;

WO 89/07136; WO 89/00201; WO 89/07136; U.S. Patent No. 4,963,489; U.S. Patent No. 5,399,346).

Methods to directly introduce nucleotide sequences *in vivo* have been attempted with formulations of DNA trapped in liposomes (Ledley *et al.*, 1987, J. Pediatrics 110:1); or in proteoliposomes that contain viral envelope receptor proteins (Nicolau *et al.*, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:1068); and DNA coupled to a polylysine-glycoprotein carrier complex. In addition, "gene guns" have been used for gene delivery into cells (Australian Patent No. 9068389). It has even been speculated that naked DNA, or DNA associated with liposomes, can be formulated in liquid carrier solutions for injection into interstitial spaces for transfer of DNA into cells (Felgner, WO90/11092).

Perhaps one of the greatest problems associated with currently devised gene therapies, whether *ex vivo* or *in vivo*, is the inability to transfer DNA efficiently into a targeted cell population and to achieve high level expression of the gene product *in vivo*. Viral vectors are regarded as the most efficient system, and recombinant replication-defective viral vectors have been used to transduce (*i.e.*, infect) cells both *ex vivo* and *in vivo*. Such vectors have included retroviral, adenovirus and adeno-associated and herpes viral vectors. While highly efficient at gene transfer, the major disadvantages associated with the use of viral vectors include the inability of many viral vectors to infect non-dividing cells; problems associated with insertional mutagenesis; inflammatory reactions to the virus and potential helper virus production, and/or production and transmission of harmful virus to other human patients.

In addition to the low efficiency of most cell types to take up and express foreign DNA, many targeted cell populations are found in such low numbers in the body that the efficiency of presentation of DNA to the specific targeted cell types is even further diminished. At present, no protocol or method, currently exists to increase the efficiency with which DNA is targeted to the targeted cell population.

3. SUMMARY OF THE INVENTION

The present invention relates to cells genetically engineered to express a target gene in response to a given "environmental cue" or "stimulus," and their use for the production of three-dimensional tissues or injectable preparations which can be used in tissue repair, replacement or enhancement, and/or for the delivery of therapeutic gene products *in vivo*. The cells used in the invention are genetically engineered to express a desired target gene in response to induction by a substance naturally produced by the

subject/recipient of the cells, or by a substance administered to the subject. The genetically engineered cells and tissues of the invention can be designed to deliver desired gene products locally (at the site of implantation) or systemically. The target gene product may be therapeutic or a detectable gene product that functions as an indicator for the subject -- i.e., indicating that medication or medical attention is required. The genetically engineered cells and tissues of the invention can be used not only to deliver gene products *in vivo* but also to improve the properties (*i.e.*, the production, processing, storage and/or use by the physician) of the cells and tissues destined for implantation *in vivo*, and most importantly, in human subjects.

The cells and tissues of the present invention are engineered to express a first gene product that responds to a given "environmental cue" or "stimulus," and in response to the "cue" or "stimulus" directly or indirectly regulates the expression of the target gene. For example, the cells and tissues of the invention can be genetically engineered to express a receptor that responds to a physiological change in the subject/recipient a ligand, and/or to a compound administered to the subject/recipient (*e.g.*, endogenous factors or hormones produced temporally by subject; exogenous factors or compounds administered to the patient to induce expression of the gene product; and physical changes or stresses such as heat shock.) The response of the receptor in turn, induces the expression of a target gene. The target gene, which may be engineered into the cell or endogenous, is controlled by a regulatory sequence (*e.g.*, a promoter, promoter element and/or enhancer) that is responsive to activation of the first gene product, *e.g.*, the receptor. The target gene product may correct, modify or regulate the physiological condition to which the cell responds. Examples include, but are not limited to engineered tissues that express target gene products which are delivered systemically (*e.g.*, secreted gene products such as growth factors, hormones, Factor VIII, Factor IX, neurotransmitters, enkaphalins) or locally secreted, intracellular or membrane bound gene products such as DNA-binding proteins involved in DNA transcription and DNA synthesis, cell-cycle-regulators, proteins involved in intracellular localization such as heat shock proteins, and membrane-bound proteins such as apolipo proteins, hormone receptors such as the insulin receptor, growth factor receptors, and ion-channel proteins. The benefit of engineering cells and tissues which express a target gene in response to a given "environmental cue" or "stimulus" is that the system is self-regulating. Alternatively, the target gene can encode a detectable gene product (*i.e.*, a reporter gene), the expression of which indicates that medical attention or medication is needed.

The genetically engineered cells can be used in an injectable preparation or to generate tissues which are used as bioreactors for the production and delivery of therapeutic gene products (*i.e.*, transcriptional or translational gene products), *in vivo* (*e.g.*, such as implants or extracorporeal devices), or *in vitro* (*e.g.*, for large-scale production and/or drug screening).

In yet another embodiment, cells genetically engineered to express a target gene in response to an "environmental cue" or "stimulus" are further engineered to generate tissues having superior properties for use *in vivo*. Examples of tissues having superior properties include, but are not limited to, engineered tissues that express gene products that reduce the inflammatory and/or immune response at the site of implantation; engineered cells which express gene products that render the tissue resistant to infection by certain pathogens; engineered cells which express gene products that modulate angiogenesis to provide the appropriate vascularization for the implanted tissues; engineered cells which express gene products with improved wound-healing properties; engineered cells which express gene products that have anti-scarring properties; and engineered cells which express a cryoprotectant or anti-desiccant to impart the product with improved storage properties for freezing, drying or freeze-drying.

The three-dimensional tissues can be prepared using genetically engineered stromal cells, parenchymal cells, or both. Techniques for achieving improved transfection efficiencies are described. In a particularly attractive embodiment, cells which normally display limited passages in culture, can be reversibly immortalized to allow for more efficient engineering -- in this embodiment, normal cells (which can be derived from the patient or subject) can be reversibly immortalized by genetically engineering the telomerase gene into the cells. The immortalized cells can be expanded, cloned and more efficiently engineered. The genetically engineered cells can then be converted back to non-immortalized cells which are more suitable for use *in vivo* by removing or disrupting the telomerase gene. The genetically engineered cells can be designed to contain, in addition to the gene of interest, a "suicide" gene that can be induced in the event the implanted cells or tissue need to be shut down or removed from the body.

The genetically engineered cells and tissues of the present invention have a wide array of applications, ranging from gene therapy, including transplantation or implantation *in vivo*, to screening cytotoxic compounds or pharmaceutical compounds *in vivo*. In particular, the engineered cells and tissues may be used to test the

cytotoxicity of vectors and other nucleic acids and their encoded gene products in gene therapy applications.

3.1. DEFINITIONS AND ABBREVIATIONS

The following terms used herein shall have the meanings indicated:

Adherent Layer: cells attached directly to the three dimensional framework or connected indirectly by attachment to cells that are themselves attached directly to the framework.

Stromal Cells: fibroblasts with or without other cells and/or elements found in loose connective tissue, including but not limited to, endothelial cells, pericytes, macrophages, monocytes, plasma cells, mast cells, adipocytes, etc.

Tissue-Specific or Parenchymal Cells: the cells which form the essential and distinctive tissue of an organ as distinguished from its supportive stromal matrix.

Three-Dimensional Framework: a three dimensional framework composed of any material and/or shape that (a) allows cells to attach to it (or can be modified to allow cells to attach to it); and (b) allows cells to grow in more than one layer. This support is inoculated with stromal cells to form the three-dimensional stromal matrix.

Three-Dimensional Stromal Matrix: a three dimensional framework which has been inoculated with stromal cells. Whether confluent or subconfluent, stromal cells according to the invention continue to grow, divide and elaborate extracellular matrix proteins. The stromal matrix will support the growth of tissue—specific cells later inoculated to form the three dimensional cell culture.

Three-Dimensional Cell Culture: a three dimensional stromal matrix which has been inoculated with tissue— specific cells and cultured. In general, the tissue specific cells used to inoculate the three-dimensional stromal matrix should include the "stem" cells (or "reserve" cells) for that tissue; i.e., those cells which generate new cells that will mature into the specialized cells that form the parenchyma of the tissue.

The following abbreviations shall have the meanings indicated:

BFU-E = burst-forming unit-erythroid
CFU-C = colony forming unit-culture
CFU-GEMM = colony forming unit-granuloid, erythroid, monocyte, megakaryocyte
EDTA = ethylene diamine tetraacetic acid
FBS = fetal bovine serum
HBSS = Hank's balanced salt solution
HS = horse serum

LTBMC = long term bone marrow culture

MEM: minimal essential medium

MHC = major histocompatibility complex

PBL = peripheral blood leukocytes

PBS = phosphate buffered saline

RPMI 1640 = Rosewell Park Memorial Institute medium number 1640 (GIBCO, Inc., Grand Island, NY)

SEM = scanning electron microscopy

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a scanning electron micrograph depicting fibroblast attachment to the three-dimensional framework and extension of cellular processes across the mesh opening. Fibroblasts are actively secreting matrix proteins and are at the appropriate stage of subconfluency which should be obtained prior to inoculation with tissue-specific cells.

FIG. 2 is a scanning electron micrograph of the three-dimensional long term bone marrow culture (LTBMC) demonstrating the 210 μm sieve area for expression of erythroid, myeloid and other colonies. Support cells have grown linearly along and enveloped the three-dimensional framework.

FIG. 3 is diagrammatic representation of the three-dimensional skin model. A dermal/epidermal junction is present, above which lies pigmented melanocytes and several layers of pigment-containing keratinocytes. The stromal cells attach to the framework and form the dermal component.

FIG. 4 is a scanning electron micrograph of the three-dimensional stroma three days after inoculation with melanocytes. Melanocytes grow normally in the three-dimensional system in that they exhibit dendrite formation, remain pigmented, and retain the ability to transfer pigment to keratinocytes.

FIG. 5 is a photomicrograph of the three-dimensional skin model grafted onto rats seven days post transplant. A distinct dermal and epidermal junction is evident. Cells show firm attachment to the mesh with no signs of rejection.

FIG. 6 is a photomicrograph of the three-dimensional skin model grafted onto rats seven days post transplant. Collagen bundles (c) and all cell types are represented, including keratinocytes (k), fibroblasts (f), adipocytes (a), and smooth muscle cells (s), arranged in a natural configuration around the nylon mesh fiber (m).

5. DETAILED DESCRIPTION OF THE INVENTION

The injectable or implantable compositions of the invention are composed, in whole or in part, of cells engineered to express a first gene product(s) that responds to a preselected "environmental cue" or "stimulus". The response of the first gene product directly or indirectly regulates the expression of one or more target genes. In accordance with this aspect of the invention, the expression of the target or second gene product is driven by a promoter activated directly or indirectly in response to the first gene product's response to a "cue" or "stimulus". The term "environmental cue" or "stimulus" used herein refers to any signal (*e.g.*, endogenous factors or hormones produced temporally by the subject; exogenous factors or compounds administered to the patient to induce expression of the target gene product; and physical changes or stress such as heat shock) that a cell can be genetically engineered to respond to. For example, the "cue" or "stimulus" may be a biological molecule (*e.g.*, a peptide, a protein, an antibody, a carbohydrate, a lipid or a nucleic acid) that binds to a receptor expressed by the engineered cells or tissues and induces the activation of a signaling cascade that results in the expression of a target gene product. Examples of target gene products include, but are not limited to, proteins such as enzymes, hormones, cytokines, antigens, antibodies, regulatory proteins, transcription proteins, receptors, and structural proteins, or nucleic acid products such as antisense RNA or ribozymes. In one embodiment, the expression of the target or second gene product corrects, modifies or regulates the physiological condition to which the engineered cell responds. For example, the cells or tissues of the present invention may be engineered to express insulin in response to changes in the level of glucose.

In a particularly useful embodiment, the engineered cells can be used to generate a skin patch that supplies the target gene product in response to a stimulus that is applied topically. For example, the cells in the patch can be engineered to express a corticosteroid receptor that activates expression of a target gene controlled by a responsive regulatory element. Target gene expression can be induced by topical appliction of a corticosteroid.

In another embodiment, the expression of the target or second gene product (*e.g.*, a reporter gene product such as β-galactosidase or green fluorescent protein (GRP)) indicates that a therapeutic or other medical attention is needed. In accordance with this embodiment, the genetically engineered cells can be used to generate an implantable "indicator" skin patch that responds to physiological changes in the recipient, and can be used to monitor diseases and disorders. For example, the cells in the patch can be engineered to express a receptor that responds to high glucose concentrations and induces expression of the target indicator gene. Thus, the implanted skin patch can be used by the recipient to monitor blood glucose levels.

In another embodiment, the cells and tissues of the present invention are genetically engineered to express multiple gene products that respond to different "environmental cues" or "stimuli", and in response, induce the expression of different target gene products. In accordance with this embodiment, the cells and tissues are genetically engineered to express target gene products under the control of different regulatory elements.

In another embodiment of the present invention, the cells and tissues are engineered to express a first gene product(s) that responds to a given "environmental cue" or "stimulus", and in response to the "cue" or "stimulus" directly or indirectly regulates the expression of a second gene product, which indicates that a compound should be taken to induce the expression of a third or target gene product. In accordance with this aspect of the present invention, the cells and tissues may be engineered to constitutively express a cell surface receptor, such as CD4, for a particular virus. When the cells become infected with the virus, *e.g.*, HIV, the expression of a viral gene product, *e.g.*, tat, induces the expression of a reporter gene, *e.g.* GFP. Detection of the expression of the reporter gene indicates that a compound that induces the expression of a peptide, protein or ribozyme that inhibits viral replication or enhances the immune system should be administered to the individual. The term "compound" as used herein refers to a naturally occurring or synthetic compound which is physiologically acceptable, can be readily administered (*i.e.*, ingested, injected or topically applied) and crosses the necessary membranes to induce the expression of a target gene product. The compound, for example, may be a pill that is ingested or an ointment that is topically applied. In a preferred embodiment, the compound is designed to specifically induce the expression of the target gene.

In a preferred embodiment, the engineered cells and tissues are capable of regulating the expression of the target gene product in an autocrine or paracrine

manner. The benefit of engineering cells or tissues that express a target gene product, which corrects, modifies, or stabilizes a physiological condition, upon receptor-ligand interaction is that the system is self-regulating -- once the stimulus is removed the cells are no longer induced to express the target gene product.

In one embodiment, the genetically engineered cells and tissues of the present invention can be used as bioreactors for the production and delivery of therapeutic and/or biologically active gene products *in vivo*, *i.e.*, in human or animal subjects. In another embodiment, the genetically engineered cells and tissues of the present invention are used *in vitro* or *in vivo* (in test animals) to screen for agents, such as pharmaceutical compounds or cytotoxic compounds, that modulate the expression of a target gene product directly or indirectly. In another embodiment, the genetically engineered cells and tissues of the present invention are used *in vitro* or *in vivo* to identify agents that modulate the activity of the target gene product directly or indirectly. Examples of agents include, but are not limited to, nucleic acids (*e.g.*, DNA and RNA), proteins, peptides, carbohydrates, lipids, antibodies, and small molecules. In yet another embodiment, the genetically engineered cells and tissues of the present invention are used scientifically to elucidate the regulation of gene expression and the signal transduction pathways activated in response to an "environmental cue" or "stimulus".

5.1 GENETICALLY ENGINEERING THE CELLS

5.1.1. THE FIRST GENE PRODUCT

In accordance with the present invention, the cells are genetically engineered to express a first gene product(s) that responds to an "environmental cue" or "stimulus", and in response induces the expression of a target gene(s). The term "first gene product" as used herein refers to any gene product that is capable of responding to an "environmental cue" or "stimulus", and in response inducing the expression of a target gene product. The first gene product may be encoded by a naturally occurring or non-naturally occurring gene product. The term "non-naturally occurring polynucleotide sequence" refers to any polynucleotide sequence that is not found in nature and is engineered by humans. This encompasses mutated polynucleotide sequences, *e.g.*, deletions, additions and/or substitutions of the wild-type polynucleotide sequence. In various embodiments, the first gene product is overexpressed, is a fusion protein, and/or a chimeric protein. The first gene product(s) may or may not be expressed by

cells normally. In a preferred embodiment, the first gene product(s) is not expressed by the cells normally.

The expression of the first gene product is controlled by constitutive or tissue-specific regulatory elements. Examples of constitutive regulatory elements, including but not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290, 304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, Cell 22, 787-797), and the herpes thymidine kinase promoter (Wagner, *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78, 1441-1445). Examples of transcriptional control regions that exhibit tissue specificity that may be utilized to regulate expression of a first gene, include but are not limited to: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, cell 38:639—646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Bid. 50:399-409; MacDonald, 1987, Hepatology 7:42S-51S); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115—122); immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647—658; Adams et al., 1985, Nature 318:533— 538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436—1444); bone specific promoters (Lian et al., 1996, Connective Tissue Res. 35:1); tissue-specific tyrosinase promoter elements (Diaz et al., 1998, J. Virol. 72: 789); muscle specific promoter elements (Baker et al., 1998, Nucleic Acids Res. 26:1092), prostate-specific enhancer elements (Schuur et al., 1996, J. Biol. Chem. 271: 7043), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268—276); alphafetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53—58); alpha—l—antitrypsin gene control region which is active in liver (Kelsey et al., 1987, Genes and Devel. 1:161—171); beta—globin gene control region which is active in myeloid cells (Magram et al., 1985, Nature 315:338—340; Kollias et al., 1986, Cell 46:89—94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703—712); myosin light chain—2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286); and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

In one embodiment, the first gene product is a receptor. The term "receptor" as used herein refers to any biological molecule(s) that is capable of specifically interacting with another biological molecule, and in response directly or indirectly activates the transcription of specifically responsive genes. A functional receptor of the

present invention may be comprised of one or more subunits. The term "functional receptor" as used herein refers to a receptor that is capable of directly or indirectly inducing the expression of a target gene product in response to receptor-ligand interaction. Examples of the receptors of the present invention include, but are not limited to, intracellular receptors such as steroid hormone receptors and cell surface receptors such as CD4, interleukin-2 receptor (IL-2R), interleukin-3 receptor (IL-3R), interleukin-4 receptor (IL-4R), interferon receptor (IFN-R), platelet-derived growth factor receptor (PDGF-R), and epidermal growth factor receptor (EGF-R). The receptor can be encoded by a naturally occurring polynucleotide sequence or a non-naturally occurring polynucleotide sequence. The term "naturally occurring polynucleotide sequence" as used herein refers to a polynucleotide sequence that is found in nature, *e.g.*, polynucleotides found in animals, unicellular organisms, microorganisms, and viruses. The term "non-naturally occurring polynucleotide sequence" refers to any polynucleotide sequence that is not found in nature and is engineered by humans.

In one embodiment of the present invention, cells and tissues are engineered to constitutively express a receptor that interacts with a particular ligand(s), and as a consequence of the receptor-ligand interaction, the expression of one or more target genes is directly or indirectly induced. In accordance with this embodiment, the expression of the target gene is driven by a promoter that is activated directly or indirectly in response to receptor-ligand interaction. The term "ligand" as used herein refers to any molecule including, but not limited to, biological molecules (*e.g.*, a peptide, a protein, a carbohydrate, lipid or nucleic acid) that interacts with a receptor. The receptor may or may not be expressed normally by the cells that are genetically engineered. In a preferred embodiment, the receptor is not expressed normally by the cells that are genetically engineered.

In a preferred embodiment, the cells and tissues of the present invention are engineered to overexpress a receptor that interacts with a particular ligand(s), and as a consequence of the receptor-ligand interaction, expression of one or more target genes is directly or indirectly induced. The overexpressed receptor may or may not expressed normally by the cells that are genetically engineered. the receptor is not expressed normally by the cells that are genetically engineered. The cells and tissues overexpressing a receptor that responds to a given "environment cue" or "stimulus" are more sensitive to the presence of a particular ligand(s). For example, cells and tissues engineered to overexpress a hormone receptor will be more sensitive to hormone and

will induce the expression of a target gene controlled by hormone response elements (HREs) in response to hormone receptor-hormone interaction. In another example, cells and tissues engineered to overexpress a cell surface receptor (*e.g.*, epidermal growth factor receptor (EGFR)) will be more sensitive to ligand and will induce the expression of a target gene controlled by a promoter activated by the MAP kinase pathway (*e.g.*, c-jun) in response to receptor-ligand interaction (*e.g.*, EGFR-EGF interaction).

In another embodiment of the present invention, cells and tissues are engineered to express a chimeric receptor comprising a ligand or compound binding domain and an activation or signaling domain. For example, cells and tissues of the present invention can be genetically engineered to express a chimeric receptor comprising a ligand or compound binding domain and a DNA binding domain. In another example, cells and tissues of the present invention can be genetically engineered to express a chimeric receptor comprising the extracellular domain of a receptor that binds to a specific ligand, a transmembrane domain of the same or a different receptor, and an intracellular domain comprising a signaling molecule. In another example, cells or tissues of the present invention are engineered express a chimeric receptor, comprising the extracellular domain of a receptor that binds to a specific ligand, a transmembrane domain of the same or a different receptor, and the intracellular domain of a different receptor. As a result of the ligand-chimeric receptor interaction, biochemical signals are generated, and transcription of a target gene is activated. The intracellular domain of a chimeric receptor will determine the signal transduction pathway activated in response to ligand-receptor interaction, and thus will affect the expression of a target gene. In accordance with this embodiment, the expression of the target gene is driven by a promoter that is activated directly or indirectly in response to ligand-chimeric receptor interaction. For example, cells or tissues of the invention can be engineered to express chimeric receptors, comprising the extracellular domain of the erythropoietin receptor (EpoR) and the intracellular domains of the IL-2 receptor (IL-2R) subunits (α, β, and δ). Cells or tissues expressing the EpoR-IL-2R chimerics will bind erythropoietin (Epo) and in response, activate the IL-2 signal transduction pathway. The expression of a target gene, whose expression regulated by a promoter containing, *e.g.*, Stat5 DNA binding elements, will be induced in response to the activation of the IL-2 signaling pathway. Techniques known to those of skill in the art can be used to generate chimeric receptors (see, *e.g.*, U.S. Patent No. 4,859,609, which is incorporated herein by reference).

In another embodiment of the present invention, cells or tissues can be engineered to express a chimeric receptor comprising an extracellular domain that binds to a specific ligand, a transmembrane domain of a receptor, and a "universal intracellular domain". The term "universal intracellular domain" as used herein refers to an intracellular domain that is capable of activating the same, specific signal transduction pathway in response to receptor-ligand interaction, regardless of the ligand. The "universal intracellular domain" is designed for optimal regulation of the transcription of a target gene. For example, the "universal intracellular domain" of the chimeric receptor may be comprised of the entire intracellular domain of the EpoR or a portion of it. Chimeric receptors comprising, the extracellular domain of IL-3R or IL-2R subunits and the intracellular domain of the EpoR will activate the same, specific signal transduction pathway in response to IL-3 or IL-2. In accordance with this embodiment, the promoter of a target gene is designed so that it is activated in response to ligand-chimeric receptor interaction.

In another embodiment of the present invention, the expression of a second or target gene product is controlled by the expression of different subunits of a receptor. In accordance with this embodiment, the sensitivity of receptor for a ligand may be regulated by expressing the high, intermediate or low affinity forms of the receptor. For example, the sensitivity of the IL-2R for IL-2 can be regulated by expressing the high affinity, heterotrimeric form ($\alpha\beta\gamma$ subunits) or the intermediate affinity, dimeric form ($\beta\gamma$ subunits) of the receptor. In another embodiment of the present invention, the expression of a target gene product is controlled by modifying the expression of a receptor that specifically responds to a ligand of interest. In accordance with this embodiment, modifications that affect the sensitivity and/or specificity of a receptor for its ligand will affect the duration and quantity of the signaling molecules activated in response to ligand-receptor interaction, and thereby affect the level of expression of a target gene. In one embodiment of the present invention, the sensitivity and specificity of a receptor of the present invention for a ligand, is modified by altering the polynucleotide sequence encoding the receptor. Examples of such alterations to the polynucleotide sequence include additions, deletions and substitutions. For example, cells and tissues may be engineered to express an EpoR mutant (*e.g.*, Epo-R G6002A; see Sokol, L. et al., 1995, Blood 86:15-22) with increased sensitivity to Epo. In another embodiment, the sensitivity of a receptor of the present invention for a ligand is modified by altering the promoter that drives the expression of the receptor. Engineered cells or tissues expressing high levels of a receptor of the present invention

will be more sensitive to its ligand. In contrast, engineered cells or tissues expressing low levels of a receptor of the present invention will be less sensitive to its ligand. In yet another embodiment, the sensitivity and/or specificity of a receptor for a ligand is modified by altering the polynucleotide sequence encoding the receptor and its promoter.

In another embodiment of the present invention, the expression of a target gene is controlled by regulating the signal transduction pathway activated in response to receptor-ligand interaction. In accordance with this aspect of the present invention, the signal transduction pathway activated in response to ligand-receptor interaction may be modified by altering the polynucleotide sequence encoding the receptor. For example, cells or tissues can be engineered to express an IL-2Rβ subunit with the serine-region deleted. This region of the IL-2Rβ has been shown to be necessary for Lck activation in response to IL-2 stimulation. Thus, in response to IL-2, cells or tissues expressing the serine-region deleted IL-2Rβ would not induce downstream signaling molecules that require Lck for activation, *e.g.*, MAPK.

In another embodiment of the present invention, the expression of a target gene is controlled by regulating the intensity and the duration of the signal transduction pathway activated in response to ligand-receptor interaction. In accordance with this aspect of the present invention, the intensity and the duration of the signal transduction pathway activated in response to ligand-receptor interaction may be modified by altering the polynucleotide sequence encoding the receptor. For example, the polynucleotide sequence encoding a receptor of the present invention may be modified to eliminate the binding site of a biological molecule that inhibits or down-regulates the signal transduced in response to ligand-receptor interaction. For example, cells or tissues may be engineered to express a mutant c-Kit receptor (*e.g.*, c-Kit with the mutationY569F; Kozlowski, M. et al., 1998, Mol Cell. Bio. 18(4):2089-2099). The SH2 domain-containing SHP-1 tyrosine phosphatase, which negatively regulates numerous mitogenic signaling pathways, has been shown to bind to tyrosine residues 569 and 567 of the c-Kit receptor. Kozlowski et al. have demonstrated that BA/F3 cells expressing c-Kit receptors with the phenylalanine substitution of c-Kit tyrosine residue 569 show a hyperproliferative response to stem cell factor.

5.1.2. EXPRESSION OF TARGET GENES

In accordance with the present invention, the expression of a target gene is induced in response to the expression or activation of the first gene product, in response

to an "environmental cue" or "stimulus". The target gene product may be a peptide or protein, such as an enzyme, hormone, cytokine, antigen, or antibody, a regulatory protein, such as a transcription factor or DNA binding protein, a structural protein, such as a cell surface protein, or the target gene product may be a nucleic acid such as a ribosome or antisense molecule. By way of example, and not by limitation, the cells and tissues of the present invention may be genetically engineered to express the following target genes:

(a) Fibroblast growth factors and derivatives thereof, including FGF-2 and bFGF (U.S. Patent No. 5,026,839); FGF-1 and Cys-modified FGF (U.S. Patent No. 5,223,483); glycosyl FGF muteins (U.S. Patent No. 5,360,896); bFGF muteins (U.S. patent No. 5,371,206); glycosyl and FGF muteins (U.S. Patent No. 5,464,943); human KGF and FGF-7 (U.S. Patent No. 4,731,170); and FGF-13 (WO98/23749);

(b) Platelet derived growth factors and derivatives, including PDGF-A and -B and heterodimeric PDGF-AB (U.S. Patent Nos. 4,766,073; 4,801,542; 5,219,759; 5,344,532; 5,605,816; and 5,665,567);

(c) Endothelial Cell Growth Factor (ECGF) and derivatives (U.S. Patent Nos. 4,868,113 and 5,227,302);

(d) Transforming Growth Factor and derivatives, TGF-α and TGF-β (U.S. Patent Nos. 4,774,412; 4,886,747; 5,482,851 and 5,633,147);

(e) Human Insulin-like Growth Factor I (IGF-1) (U.S. Patent Nos. 4,963,665 and 5,070,075);

(f) Human Growth Hormone (hGH) and derivatives (U.S. Patent Nos. 3,853,832; 3,853,833; and 5,597,709);

(g) Clotting Factors (U.S. Patent No. 4,757,006);

(h) Erythropoietin (EPO) (U.S. Patent No. 4,703,008); and

(i) Human insulin and analogs, preproinsulin (U.S. Patent No. 4,431,740), insulin analogs (U.S. Patent Nos. 4,701,440, 5,008,241, 5,149,777, 5,164,366, 5,324,641, 5,618,913, 5,716,927), superactive insulin analogs (4,992,417) and insulin precursor analogs (U.S. Patent No. 5,324,641).

(j) Trehalose, yeast trehalose biosynthetic genes (U.S. Patent No. 5,433,254), maltose-trehalose converting enzyme (U.S. Patent No. 5,736,380).

In one embodiment, the target gene product is encoded by naturally occurring polynucleotide sequences, *e.g.*, polynucleotide sequences found in microorganisms, unicellular organisms, viruses, and animals, preferably humans. In a preferred embodiment, the target gene product is encoded by non-naturally occurring

polynucleotide sequences and is specifically designed to be stable *in vivo*. The term "non-naturally occurring polynucleotide sequences" encompasses polynucleotide sequences found in nature which have been modified (*i.e.*, mutated polynucleotide sequences -- having deleted sequences. additions to the sequence, and/or substitutions in the sequence). In various embodiments. a target gene(s) encodes a fusion protein(s) and/or chimeric protein(s).

In one embodiment of the present invention, the cells and tissues are genetically engineered to express target genes coding for gene products which have a therapeutic effect, *e.g.*, differentiation factors, growth factors, hormones, receptors and enzymes. In another embodiment, the cells and tissues are genetically engineered to express target genes coding for gene products that have an inhibitory effect, *e.g.*, ribozyme, antisense molecules and triple helices.

In another embodiment, the target gene encodes a detectable indicator gene product. Expression of the indicator gene product can be used to alert the subject that medical attention, or administration of the medicine is required.

The regulatory elements operably linked to the target gene(s) bind to transcription factors activated, directly or indirectly, in response to the expression or activation of a first gene product(s). Examples of regulatory promoters induced in response to a specific stimuli, in this the expression of a first gene product, include tetracycline responsive elements, nicotine responsive elements, insulin responsive element, glucose responsive elements, interferon responsive elements, glucocorticoid responsive elements estrogen/progesterone responsive elements, retinoid acid responsive elements, viral transactivators, early or late promoter of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the promoter for 3-phosphoglycerate and the promoters of acid phosphatase, and the regulatory sequences of the metallothionein gene (Brinster, *et al.*, 1982, Nature 296, 39-42). In addition, artificial response elements could be constructed, composed of multimers of transcription factor binding sites and hormone-response elements similar to the molecular architecture of naturally-occurring promoters and enhancers (*e.g.*, see Herr, W & Clarke, J Cell (1986) 45(3): 461-70). Such artificial composite regulatory regions could be designed to respond to any desirable signal and be expressed in particular cell-types depending on the promoter/enhancer binding sites selected.

5.1.3. ADDITIONAL GENES EXPRESSED BY CELLS GENETICALLY ENGINEERED TO BE RESPONSIVE

TO CUES AND STIMULUS

In a preferred embodiment, cells genetically engineered to express first gene products and target gene products are further engineered to express gene products which provide enhanced properties to the genetically engineered cells. In one embodiment, cells are engineered to further express gene products which enhance cell growth, *e.g.*, fibroblast growth factors (FGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor (TGF). In a preferred embodiment of the present invention, the cells and tissues are genetically engineered to further express gene products which alter the differentiation pathway of the cells. In such an embodiment, cells, in particular stem cells, are engineered to express intracellular or secreted proteins, *e.g.*, DNA binding proteins, transcription factors, growth factors, hormones, cytokines, which mediate cellular interactions which influence the differentiation pathway of the cells. In another embodiment, the cells may be engineered to express a suicide gene product on cue, *e.g.*, thymidine kinase.

In a preferred embodiment of the present invention, the cells and tissues are genetically engineered to express a gene product which results in the immortalization of the cells. Such an embodiment is particularly attractive for cells which normally display limited passages in culture, such as fibroblasts. In accordance with the present invention, these cells can be reversibly immortalized to allow for more efficient engineering. In this embodiment, cells in culture or cells derived from a patient may be reversibly immortalized by genetically engineering the telomerase gene or an oncogene into the cells. The immortalized cells can be expanded cloned and more efficiently engineered. The genetically engineered cells can then be converted back to the non-immortalized phenotype which is more appropriate for *in vivo* use by disrupting or removing the telomerase gene or oncogene. In one embodiment of the present invention, the telomerase gene is engineered between activatable recombination sites, such as *loxP* sites, which may be activated to excise the telomerase gene. Further backup safeguards may be built in to ensure that the telomerase gene is inactivated, including, but not limited to, antibiotic resistance genes and suicide genes which may be engineered into the system to ensure reliable reversible immortality.

In another embodiment, the cells and tissues of the present invention are genetically engineered to further express gene products which modulate vascularization, such as vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF) and basic FGF; angiogenesis factors, and anti-angiogenesis factors.

In another preferred embodiment, the cells and tissues of the present invention are genetically engineered to further express gene products which provide protective functions *in vitro* such as cyropreservation and anti-desiccation properties, *e.g.*, trechalose (U.S. Patent Nos. 4,891,319; 5,290,765; 5,693,788). The cells and tissues of the present invention may also be engineered to express gene products which provide a protective function *in vivo*, such as those which would protect the cells from an inflammatory response and protect against rejection by the host's immune system, such as HLA epitopes, major histo-compatibility epitopes, immuno-globulin and receptor epitopes, epitopes of cellular adhesion molecules, cytokines, chemokines. Alternatively, cells and tissues may be protected against rejection by the host's immune system by mutating or deleting genes encoding proteins that identify the cells or tissues as non-self. The cells and tissues of the present invention may be engineered to express gene products such as TGF-β, including TGFβ-1, TGFβ-2, TGFβ-3, TGFβ-4 and TGFβ-5, which regulate growth and differentiation and accelerate wound healing (Noda et al. 1989, Endocrin. 124: 2991-2995; Goey et al. 1989, J. Immunol. 143: 877-880, Mutoe et al. 1987, Science 237: 1333-1335). Cells and tissues of the present invention may be engineered to express mitogens, such as PDGF which increase the rate of cellularity and granulation in tissue formation (Kohler et al. 1974, Exp. Cell. Res. 87: 297-301).

By way of example, and not by limitation, the cells and tissues of the present invention may be genetically engineered to express nucleotide sequences or fragments thereof of the following genes:

(a) Fibroblast growth factors and derivatives thereof, including FGF-2 and bFGF (U.S. Patent No. 5,026,839); FGF-1 and Cys-modified FGF (U.S. Patent No. 5,223,483); glycosyl FGF muteins (U.S. Patent No. 5,360,896); bFGF muteins (U.S. patent No. 5,371,206); glycosyl and FGF muteins (U.S. Patent No. 5,464,943); human KGF and FGF-7 (U.S. Patent No. 4,731,170); and FGF-13 (WO98/23749);

(b) Platelet derived growth factors and derivatives, including PDGF-A and -B and heterodimeric PDGF-AB (U.S. Patent Nos. 4,766,073; 4,801,542; 5,219,759; 5,344,532; 5,605,816; and 5,665,567);

(c) Endothelial Cell Growth Factor (ECGF) and derivatives (U.S. Patent Nos. 4,868,113 and 5,227,302);

(d) Transforming Growth Factor and derivatives, TGF-α and TGF-β (U.S. Patent Nos. 4,774,412; 4,886,747; 5,482,851 and 5,633,147);

(e) Human Insulin-like Growth Factor I (IGF-1) (U.S. Patent Nos. 4,963,665 and 5,070,075);

(f) Human Growth Hormone (hGH) and derivatives (U.S. Patent Nos. 3,853,832; 3,853,833; and 5,597,709);

(g) Clotting Factors (U.S. Patent No. 4,757,006);

(h) Erythropoietin (EPO) (U.S. Patent No. 4,703,008); and

(i) Human insulin and analogs, preproinsulin (U.S. Patent No. 4,431,740), insulin analogs (U.S. Patent Nos. 4,701,440, 5,008,241, 5,149,777, 5,164,366, 5,324,641, 5,618,913, 5,716,927), superactive insulin analogs (4,992,417) and insulin precursor analogs (U.S. Patent No. 5,324,641).

(j) Trehalose, yeast trehalose biosynthetic genes (U.S. Patent No. 5,433,254), maltose-trehalose converting enzyme (U.S. Patent No. 5,736,380).

In accordance with the present invention, there are a number of ways that the gene products may be engineered to be expressed by the cells and tissues of the present invention. The gene products may be engineered to be expressed constitutively or in a tissue-specific or stimuli-specific manner. In accordance with this aspect of the invention, the nucleotide sequences encoding gene products may be operably linked to promoter elements which are constitutively active, tissue-specific or induced upon presence of a specific stimuli.

Examples of regulatory promoter elements include tetracycline responsive elements, nicotine responsive elements, insulin responsive element, glucose responsive elements, interferon responsive elements, glucocorticoid responsive elements estrogen/progesterone responsive elements, retinoid acid responsive elements, viral transactivators, early or late promoter of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the promoter for 3-phosphoglycerate and the promoters of acid phosphatase. In addition, artificial response elements could be constructed, composed of multimers of transcription factor binding sites and hormone-response elements similar to the molecular architecture of naturally-occurring promoters and enhancers (*e.g.*, see Herr, W & Clarke, J Cell (1986) 45(3): 461-70). Such artificial composite regulatory regions could be designed to respond to any desirable signal and be expressed in particular cell-types depending on the promoter/enhancer binding sites selected.

In accordance with the present invention, transcriptional control regions that exhibit tissue specificity that may be utilized to regulate expression of a gene, include but are not limited to: elastase I gene control region which is active in pancreatic acinar

cells (Swift et al., 1984, cell 38:639—646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Bid. 50:399-409; MacDonald, 1987, Hepatology 7:42S-51S); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115—122); immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647—658; Adams et al., 1985, Nature 318:533— 538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436—1444); bone specific promoters (Lian et al., 1996, Connective Tissue Res. 35:1); tissue-specific tyrosinase promoter elements (Diaz et al., 1998, J. Virol. 72: 789); muscle specific promoter elements (Baker et al., 1998, Nucleic Acids Res. 26:1092), prostate-specific enhancer elements (Schuur et al., 1996, J. Biol. Chem. 271: 7043), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268—276); alphafetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53—58); alpha—1—antitrypsin gene control region which is active in liver (Kelsey et al., 1987, Genes and Devel. 1:161—171); beta—globin gene control region which is active in myeloid cells (Magram et al., 1985, Nature 315:338—340; Kollias et al., 1986, Cell 46:89—94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703—712); myosin light chain—2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286); and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

In addition, such promoters can be inducible or constitutive, including but not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290, 304-310), the promoter contained in the 3′ long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, Cell 22, 787-797), the herpes thymidine kinase promoter (Wagner, *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78, 1441-1445), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, 1982, Nature 296, 39-42), etc.

5.2. ENGINEERING THE CELLS

The genes of the invention (*i.e.*, the first gene(s), the target gene(s), and additional genes) may be introduced into the cell using recombinant DNA technology well known in the art. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the target gene coding sequences and appropriate transcriptional and translational control signals.

These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and PCR, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, supra, and Ausubel et al., 1989, supra. Alternatively, DNA capable of encoding the target gene product sequences may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

A variety of host-expression vector systems may be utilized to express gene(s) (*i.e.*, the first gene(s), the target gene(s), and additional genes) coding sequences of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the target gene product of the invention *in situ*. These include but are not limited to mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the gene coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing target gene product in infected hosts. (*e.g.*, See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted target gene product coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the target gene coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both

natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

For long term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the first gene product may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. Cell lines can then be analyzed for optimal expression levels of the target gene product. This method may advantageously be used to engineer cell lines which express the target gene products. Such cell lines would be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the target gene product.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hydro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

In another embodiment of the present invention, the cells and tissues may be engineered to express an endogenous gene under the control of a heterologous regulatory element, in which case the regulatory sequences of the endogenous gene may be replaced by homologous recombination, *e.g.*, see Chappell, U.S. Patent No. 5,272,071; PCT Publication No. WO91/06667, published May 16, 1991, which are

incorporated by reference herein. As described herein, gene targeting can be used to replace a gene's existing regulatory region with a regulatory sequence isolated from a different gene or a novel regulatory sequence synthesized by genetic engineering methods. Such regulatory sequences may be comprised of promoters, enhancers, scaffold-attachment regions, negative regulatory elements, transcriptional initiation sites, regulatory protein binding sites or combinations of said sequences. Alternatively, sequences which affect the structure or stability of the RNA or protein produced may be replaced, removed, added, or otherwise modified by targeting, including polyadenylation signals, mRNA stability elements, splice sites, leader sequences for enhancing or modifying transport or secretion properties of the protein, or other sequences which alter or improve the function or stability of protein or RNA molecules.

The targeting event may be a simple insertion of the regulatory sequence, placing the gene under the control of the new regulatory sequence, *e.g.*, inserting a new promoter or enhancer or both upstream of a gene. Alternatively, the targeting event may be a simple deletion of a regulatory element, such as the deletion of a tissue-specific negative regulatory element. Alternatively, the targeting event may replace an existing element; for example, a tissue-specific enhancer can be replaced by an enhancer that has broader or different cell-type specificity than the naturally occurring elements. Here, the naturally occurring sequences are deleted and new sequences are added. In all cases, the identification of the targeting event may be facilitated by the use of one or more selectable marker genes that are contiguous with the targeting DNA, allowing for the selection of cells in which the exogenous DNA has integrated into the host cell genome. The identification of the targeting event may also be facilitated by the use of one or more marker genes exhibiting the property of negative selection, such that the negatively selectable marker is linked to the exogenous DNA, but configured such that the negatively selectable marker flanks the targeting sequence, and such that a correct homologous recombination event with sequences in the host cell genome does not result in the stable integration of the negatively selectable marker. Markers useful for this purpose include the Herpes Simplex Virus thymidine kinase (TK) gene or the bacterial xanthine-guanine phosphoribosyl-transferase (gpt) gene.

5.2.1. CELLS TO BE GENETICALLY ENGINEERED

In accordance with the present invention, a variety of primary or secondary cells or cell strains may be used, including stromal and parenchymal cells. Further, the cells may be either fibroblasts, epithelial cells, keratinocytes, chondrocytes, hepatocytes,

mesenchymal cells, chondro progenitor cells, or parenchymal cells of various tissues, including stem cells. A number of cell types may be used in accordance with the present invention, including but not limited to cells isolated from skin, bone marrow, liver, pancreas, kidney, adrenal and neurological tissue to name a few. Other cell types that may be used in accordance with the present invention are immune cells, macrophages/ monocytes, adipocytes, pericytes, reticular cells etc. In a further embodiment, secondary cell lines may be used as engineered responsive cells and tissues in accordance with the present invention, including, but not limited to hepatic cell lines, such as Chang liver cells, or other cell lines such as CHO, VERO, BHK, Hela, COS, MDCK, 293, 373, and W138 cell lines.

The engineered cells and tissues of the present invention may be obtained from an animal, preferably a mammal, and most preferably a human being. In a preferred embodiment, the cells or tissues used for transplantation or implantation *in vivo* are obtained from the same species that will receive them. In another embodiment, the cells and tissues of the present invention may be autologous or non-autologous to the patient receiving them. In a preferred embodiment, the cells and tissues are autologous to the patient receiving them.

Stromal cells such as these fibroblasts may be derived from organs such as skin, liver, pancreas, etc. which can be obtained by biopsy (where appropriate). Parenchymal cells may be obtained from cell suspensions prepared by disaggregating the desired tissue using standard techniques. Again, where the engineered cells and tissues are to be used for transplantation or implantation *in vivo*, it is preferable to obtain the stromal or parenchymal cells from the patient's own tissues.

Although not all cells that may be used in accordance with the present invention grow in monolayers, such as fibroblasts, but rather grow in suspension, such as migratory immune cells. These cell types can be cultured using a pre-established three-dimensional cell culture system as described in U.S. Patent No. 5,785,964, incorporated by reference herein in its entirety. The stromal support matrix comprises stromal cells, such as fibroblasts, actively growing on a three-dimensional matrix. Stromal cells may be also include other cells found in loose connective tissue such as endothelial cells, macrophages, monocytes, adipocytes, pericytes, reticular cells found in bone marrow, etc. The stromal matrix provides the support, growth factors and regulatory factors necessary to sustain long-term a time proliferation of cells in culture. When grown in this three-dimensional system, the proliferating cells mature and segregate properly to form components of adult tissues analogous to counterparts found *in vivo*.

The cells of the present invention may be engineered to express one or more target gene sequences of interest, and may then be introduced into a patient in appropriate positions. Alternatively, cells that express an unimpaired target gene of interest and that are from a major histocompatibility complex ("MHC") matched individual can be utilized, and may include, for example, parenchymal cells. The expression of the gene sequences is controlled by the appropriate gene regulatory sequences to allow such expression in the necessary cell types. Such gene regulatory sequences are well known to the skilled artisan. Such cell-based gene therapy techniques are well known to those skilled in the art, see, *e.g.*, Anderson, U.S. Patent No. 5,399,349.

When the cells to be administered are non-autologous cells, they can be administered using well known techniques that prevent a host immune response against the introduced cells from developing. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

5.2.2. SELECTION OF THE GENETICALLY ENGINEERED CELLS AND TISSUES

The genetically engineered cells and tissues of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. Expression of a gene(s) of the present invention can be readily detected, *e.g.*, by quantifying protein and/or RNA. Many methods standard in the art can be thus employed, including but not limited to kinase assays, immunoassays to detect and/or visualize gene expression (*e.g.*, Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect gene expression by detecting and/or visualizing respectively mRNA encoding a gene (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc), etc. The activity of a target gene product can be tested by assessing the effect of the tissue culture supernatant from the genetically engineered cells or tissues on different cells in culture. Known effects of the target gene, such as the activation of a signaling molecule or the induction of a gene product, can be assessed by standard methods known to those of skill in the art.

Cells and tissues for use *in vivo* can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows,

monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used.

5.3. PRODUCTION OF TISSUES USING GENETICALLY ENGINEERED CELLS

The engineered cells can be used, with or without other cells or cell types which may or may not be genetically engineered: (a) to produce three-dimensional tissues *in vitro* (for implantation *in vivo*, or for use as extracorporeal devices or for use *in vitro*, as bioreactors or); (b) to produce three-dimensional tissues *in vivo* (*e.g.*, by inoculating the cells onto a scaffold implanted *in vivo*); or (c) alternatively, the cells can be formulated as an injectable preparation of cells mixed with extracellular matrix proteins, collagen or synthetic crosslinkable polymers.

5.3.1. THREE-DIMENSIONAL TISSUE CULTURES

In the most preferred embodiment, the genetically engineered cells are grown on a three-dimensional framework to produce three-dimensional cultures analogous to tissue counterparts *in vivo* using the techniques described in the U.S. Patent No.s. 4,721,096; 4,963,489; and 5,443,950 each of which is incorporated by reference herein in its entirety.

In this system, a living stromal tissue is produced *in vitro* composed of stromal cells, such as fibroblasts, actively growing on a three-dimensional framework. In accordance with this embodiment gentically engineered may be mixed with other cells including other cells found in loose connective tissue such as endothelial cells, macrophages/monocytes, adipocytes, pericytes, reticular cells found in boar marrow stroma etc. The three-dimensional stromal cell culture provides the support, growth factors, and regulatory factors necessary to sustain long term active proliferation of cells in culture. When grown in this three-dimensional system, the proliferating cells mature and segregate to form components of adult tissues analogous to counterparts *in vivo*. This system is particularly advantageous for producing tissues for use in human subjects, since human stromal cells can be used to create a "human tissue" -- *i.e.*, the cells and the extracellular matrix proteins generated in the three-dimensional stromal tissue will be of human origin and less likely rejected by patient-recipients of the engineered tissue.

This embodiment of the invention is based, in part, on the discovery that growth of stromal cells in three dimensions will sustain active proliferation of cells in culture

for longer periods of time than will monolayer systems. This may be due, in part, to the increased surface area of the three-dimensional framework which results in a prolonged period of subconfluency and active proliferation of stromal cells. These proliferating stromal cells elaborate proteins (*e.g.*, extracellular matrix proteins which form the connective tissue of the stromal tissue), growth factors and regulatory factors necessary to support the long term proliferation of both stromal cells (and parenchymal cells) inoculated onto the stromal tissue. In addition, the three-dimensionality of the framework allows for a spatial distribution which more closely approximates conditions *in vivo*, thus allowing for the formation of microenvironments conducive to cellular maturation and migration.

In this system, stromal cells, such as human fibroblasts, are grown on a three-dimensional framework composed of any material and/or shape that: (a) allows cells to attach to it (or can be modified to allow cells to attach to it); and (b) allows cells to grow in more than one layer. A number of different materials may be used to form the framework, including but not limited to: nylon (polyamides), dacron (polyesters), polystyrene, polypropylene, polyacrylates, polyvinyl compounds (*e.g.*, polyvinylchloride), polycarbonate (PVC), polytetrafluorethylene (PTFE; teflon), thermanox (TPX), nitrocellulose, cotton, polyglycolic acid (PGA), cat gut sutures, collagen, cellulose, gelatin, dextran, etc. Any of these materials may be woven into a mesh, for example, to form the three-dimensional matrix. Certain materials, such as nylon, polystyrene, etc., are poor substrates for cellular attachment. When these materials are used as the three-dimensional support framework, it is advisable to pre-treat the framework prior to inoculation of stromal cells in order to enhance the attachment of stromal cells to the matrix. For example, prior to inoculation with stromal cells, nylon matrices could be treated with 0.1 M acetic acid, and incubated in polylysine, FBS, and/or collagen to coat the nylon. Polystyrene could be similarly treated using sulfuric acid.

Where the three-dimensional culture is itself to be implanted *in vivo*, it may be preferable to use biodegradable materials such as polyglycolic acid, catgut suture material, collagen (such as collagen sponge), or gelatin, for example. Where the cultures are to be maintained for long periods of time or crypreserved, nondegradable materials such as nylon, dacron, polystyrene, polyacrylates, polyvinyls, teflons, cotton, etc. may be preferred. A convenient nylon mesh which could be used in accordance with the invention is Nitex, a nylon filtration mesh having an average pore size of 210 μm and an average nylon fiber diameter of 90 μm (#3—210/36, Tetko, Inc., N.Y.).

Stromal cells comprising fibroblasts, with or without other cells and elements described below, are inoculated onto the framework. These fibroblasts may be derived from organs, such as skin, liver, pancreas, etc. which can be obtained by biopsy (where appropriate) or upon autopsy. In fact fibroblasts can be obtained in quantity rather conveniently from any appropriate cadaver organ. As previously explained, fetal fibroblasts can be used to form a "generic" three-dimensional stromal matrix that will support the growth of a variety of different cells and/or tissues. However, a "specific" stromal matrix may be prepared by inoculating the three-dimensional framework with fibroblasts derived from the same type of tissue to be cultured and/or from a particular individual who is later to receive the cells and/or tissues grown in culture in accordance with the three-dimensional system of the invention.

Fibroblasts may be readily isolated by disaggregating an appropriate organ or tissue which is to serve as the source of the fibroblasts. This may be readily accomplished using techniques known to those skilled in the art. For example, the tissue or organ can be disaggregated mechanically and/or treated with digestive enzymes and/or chelating agents that weaken the connections between neighboring cells making it possible to disperse the tissue into a suspension of individual cells without appreciable cell breakage. Enzymatic dissociation can be accomplished by mincing the tissue and treating the minced tissue with any of a number of digestive enzymes either alone or in combination. These include but are not limited to trypsin, chymotrypsin, collagenase, elastase, and/or hyaluronidase, DNase, pronase, dispase etc. Mechanical disruption can also be accomplished by a number of methods including, but not limited to the use of grinders, blenders, sieves, homogenizers, pressure cells, or insonator name but a few. For a review of tissue disaggregation techniques, see Freshney, Culture of Animal Cells. A Manual of Basic Technique, 2d Ed., A.R. Liss, Inc., New York, 1987, Ch. 9, pp. 107-126.

Once the tissue has been reduced to a suspension of individual cells, the suspension can be fractionated into subpopulations from which the fibroblasts and/or other stromal cells and/or elements can be obtained. This also may be accomplished using standard techniques for cell separation including but not limited to cloning and selection of specific cell types, selective destruction of unwanted cells (negative selection), separation based upon differential cell agglutinability in the mixed population, freeze-thaw procedures, differential adherence properties of the cells in the mixed population, filtration, conventional and zonal centrifugation, centrifugal elutriation (counterstreaming centrifugation), unit gravity separation, countercurrent

distribution, electrophoresis and fluorescence-activated cell sorting. For a review of clonal selection and cell separation techniques, see Freshney, Culture of Animal Cells. A Manual of Basic Techniques, 2d Ed., A.R. Liss, Inc., New York, 1987, Ch. 11 and 12, pp. 137-168.

The isolation of fibroblasts may, for example, be carried out as follows: fresh tissue samples are thoroughly washed and minced in Hanks balanced salt solution (HBSS) in order to remove serum. The minced tissue is incubated from 1-12 hours in a freshly prepared solution of a dissociating enzyme such as trypsin. After such incubation, the dissociated cells are suspended, pelleted by centrifugation and plated onto culture dishes. All fibroblasts will attach before other cells, therefore, appropriate stromal cells can be selectively isolated and grown. The isolated fibroblasts can then be grown to confluency, lifted from the confluent culture and inoculated onto the three-dimensional framework (see, Naughton et al., 1987, J. Med. 18(3&4):219-250). Inoculation of the three-dimensional framework with a high concentration of stromal cells, *e.g.*, approximately 10^6 to 5 x 10^7 cells/ml, will result in the establishment of the three-dimensional stromal support in shorter periods of time.

In addition to fibroblasts, other cells may be added to form the three-dimensional stromal matrix required to support long term growth in culture. For example, other cells found in loose connective tissue may be inoculated onto the three-dimensional support along with fibroblasts. Such cells include but are not limited to endothelial cells, pericytes, macrophages, monocytes, plasma cells, mast cells, adipocytes, etc. These stromal cells may readily be derived from appropriate organs such as skin, liver, etc., using methods known in the art such as those discussed above. In one embodiment of the invention, stromal cells which are specialized for the particular tissue to be cultured may be added to the fibroblast stroma. For example, stromal cells of hematopoietic tissue, including but not limited to fibroblasts, endothelial cells, macrophages/monocytes, adipocytes and reticular cells, could be used to form the three-dimensional subconfluent stroma for the long term culture of bone marrow *in vitro*. Hematopoietic stromal cells may be readily obtained from the "buffy coat" formed in bone marrow suspensions by centrifugation at low forces, *e.g.*, 3000 x g. Stromal cells of liver may include fibroblasts, Kupffer cells, and vascular and bile duct endothelial cells. Similarly, glial cells could be used as the stroma to support the proliferation of neurological cells and tissues: glial cells for this purpose can be obtained by trypsinization or collagenase digestion of embryonic or adult brian

(Ponten and Westermark, 1980, in Federof. S. Hertz. L., eds, "Advances in Cellular Neurobiology," Vol.1, New York, Academic Press, pp. 209-227).

Again, where the cultured cells are to be used for transplantation or implantation *in vivo* it is preferable to obtain the stromal cells from the patient's own tissues. The growth of cells in the presence of the three-dimensional stromal framework may be further enhanced by adding to the framework, or coating the framework with proteins (*e.g.*, collagens, elastic fibers, reticular fibers) glycoproteins, glycosaminoglycans (*e.g.*, heparin sulfate, chondroitin-4—sulfate, chondroitin—6-sulfate, dermatan sulfate, keratin sulfate, etc.), cellular matrix, and/or other materials.

After inoculation of the stromal cells, the three- dimensional culture should be incubated in an appropriate nutrient medium. Many commercially available media such as RPMI 1640, Fisher's, Iscove's, Mccoy's, and the like may be suitable for use. It is important that the three- dimensional stromal culture be suspended or floated in the medium during the incubation period in order to maximize proliferative activity. In addition, the culture should be "fed" periodically to remove the spent media, depopulate released cells, and add fresh media.

During the incubation period, the stromal cells grow linearly along and envelop the three-dimensional framework before beginning to grow into the openings, and elaborate growth factors and regulatory factors necessary to support the long term proliferation of the cells inoculated or the framework, as well as other proteins, such as collagen and other extracellular matrix proteins that form the stromal matrix. The resulting stromal tissue can be used in vivo or may be additionally inoculated on the framework, as well as other proteins, such as collagen and other extracellular matrix proteins that form the stromal matrix. The resulting stromal tissue can be used *in vivo* or may be additionally inoculated with parenchymal cells to produce a tissue analogous to tissue counterparts *in vivo*.

The openings of the framework should be of an appropriate size to allow the stromal cells to stretch across the openings. Maintaining actively growing stromal cells which stretch across the framework enhances the production of growth factors which are elaborated by the stromal cells, and hence will support long term cultures. For example, if the openings are too small, the stromal cells may rapidly achieve confluence, exhibit contact inhibition and cease production of the appropriate factors necessary to support proliferation and maintain long term cultures. If the openings are too large, the stromal cells may be unable to stretch across the opening; this will also decrease stromal cell production of the appropriate factors necessary to support

proliferation and maintain long term cultures. When using a mesh type of framework, as exemplified herein, we have found that openings ranging from about 150 μm to about 220 μm will work satisfactorily. However, depending upon the three—dimensional structure and intricacy of the framework, other sizes may work equally well. In fact, any shape or structure that allow the stromal cells to stretch and continue to replicate and grow for lengthy time periods will work in accordance with the invention.

Different proportions of the various types of collagen deposited on the framework can affect the growth of the later inoculated parenchymal cells. For example, for optimal growth of hematopoietic cells, the framework should preferably contain collagen types III, IV and I in an approximate ratio of 6:3:1 in the initial matrix. For three-dimensional skin culture systems, collagen types I and III are preferably deposited in the initial framework. The proportions of collagen types deposited can be manipulated or enhanced by selecting fibroblasts which elaborate the appropriate collagen type. This can be accomplished using monoclonal antibodies of an appropriate isotype or subclass that is capable of activating complement, and which define particular collagen types. These antibodies and complement can be used to negatively select the fibroblasts which express the desired collagen type. Alternatively, the stromal cells used to inoculate the framework can be a mixture of cells which synthesize the appropriate collagen types desired. The distribution and origins of the various types of collagen is shown in Table I. Thus, depending upon the tissue to be cultured and the collagen types desired, the appropriate stromal cell(s) may be selected to inoculate the three-dimensional framework.

TABLE I

DISTRIBUTIONS AND ORIGINS OF VARIOUS TYPES OF COLLAGEN

Collagen Type	Principal Tissue Distribution	Cells of Origin
I	Loose and dense ordinary connective tissue; collagen fibers	Fibroblasts and reticular cells; smooth muscle cells
	Fibrocartilage	
	Bone	Osteoblast
	Dentin	Odontoblasts

II	Hyaline and elastic cartilage	Chondrocytes
	Vitreous body of eye	Retinal cells
III	Loose connective tissue; reticular fibers	Fibroblasts and reticular cells
	Papillary layer of dermis	
	Blood vessels	Smooth muscle cells; endothelial cells
IV	Basement membranes	Epithelial and endothelial cells
	Lens capsule of eye	Lens fibers
V	Fetal membranes; placenta	Fibroblast
	Basement membranes	
	Bone	
	Smooth muscle	Smooth muscle cells
		Fibroblasts
VI	Connective Tissue	
VII	Epithelial basement membranes, anchoring fibrils	Fibroblasts, keratinocytes
	Cornea	Corneal fibroblasts
VIII	Cartilage	
IX	Hypertrophic cartilage	
X	Cartilage	
		Fibroblasts
XI	Papillary dermis	Fibroblasts
XII	Reticular dermis	Fibroblasts
XIV, undulin	P170 bullous pemphigoid antigen	Keratinocytes
XVII		

During incubation of the three-dimensional culture, proliferating cells may be released from the framework. These released cells may stick to the walls of the culture vessel where they may continue to proliferate and form a confluent monolayer. This should be prevented or minimized, for example, by removal of the released cells during

feeding, or by transferring the three-dimensional stromal framework to a new culture vessel. The presence of a confluent monolayer in the vessel will "shut down" the growth of cells in the three-dimensional culture. Removal of the confluent monolayer or transfer of the three-dimensional culture framework to fresh media in a new vessel will restore proliferative activity of the three-dimensional culture system. Such removal or transfers should be done in any culture vessel which has a stromal monolayer exceeding 25% confluency. Alternatively, the culture system could be agitated to prevent the released cells from sticking, or instead of periodically feeding the cultures, the culture system could be set up so that fresh media continuously flows through the system. The flow rate could be adjusted to both maximize proliferation within the three-dimensional culture, and to wash out and remove cells released from the matrix, so that they will not stick to the walls of the vessel and grow to confluence. In any case, the released stromal cells can be collected and cryopreserved for future use.

In alternative embodiments, the genetically engineered cells can be used to produce tissue equivalents using other systems known in the art, including but not limited to using synthetic scaffolding support systems (*e.g.*, see U.S. Patent Nos. 5,770,193; 5,770,417; 5,736,372 each of which is incorporated by reference in its entirety); bovine collagen matrices (*e.g.* see U.S. Patent No. 4,485,096 incorporated herein by reference in its entirety), cross-linked collagen sponge matrices (*e.g.*, see PCT WO 91/16010, incorporated herein by reference in its entirety) or any other equivalent method or technique known to those of skill in the art.

5.3.2. INJECTIBLE AND IMPLANTABLE PREPARATIONS

In one embodiment genetically engineered cells are grown on extracellular matrix preparations composed of stromal cells which provide a three-dimensional cell culture system which may be formulated as an injectable, see PCT WO 96/39101, incorporated herein by reference in its entirety. In an alternative embodiment, the cells and tissues of the present invention are grown using polymerizable or cross linking hydrogels as described in U.S. Patent Nos. 5,709,854; 5,516,532; 5,654,381; and WO98/52543, each of which is incorporated herein by reference in their entirety. Examples of materials which can be used to form a hydrogel include modified alginates. Alginate is a carbohydrate polymer isolated from seaweed, which can be cross-linked to form a hydrogel by exposure to a divalent cation such as calcium, as described, for example in WO 94/25080, the disclosure of which is incorporated herein by reference. Alginate is ionically cross-linked in the presence of divalent cations, in

water, at room temperature, to form a hydrogel matrix. As used herein, the term "modified alginates" refers to chemically modified alginates with modified hydrogel properties.

Additionally, polysaccharides which gel by exposure to monovalent cations, including bacterial polysaccharides, such as gellan gum, and plant polysaccharides, such as carrageenans, may be cross-linked to form a hydrogel using methods analogous to those available for the cross-linking of alginates described above.

Modified hyaluronic acid derivatives are particularly useful. As used herein, the term "hyaluronic acids" refers to natural and chemically modified hyaluronic acids. Modified hyaluronic acids may be designed and synthesized with preselected chemical modifications to adjust the rate and degree of cross-linking and biodegradation.

Covalently cross-linkable hydrogel precursors also are useful. For example, a water soluble polyamine, such as chitosan, can be cross-linked with a water soluble diisothiocyanate, such as polyethylene glycol diisothiocyanate.

Alternatively, polymers may be utilized which include substituents which are cross-linked by a radical reaction upon contact with a radical initiator. For example, polymers including ethylenically unsaturated groups which can be photochemically cross-linked which may be utilized, as disclosed in WO 93/17669, the disclosure of which is incorporated herein by reference. In this embodiment, water soluble macromers that include at least one water soluble region, a biodegradable region, and at least two free radical-polymerizable regions, are provided. Examples of these macromers are PEG-oligolactyl-acrylates, wherein the acrylate groups are polymerized using radical initiating systems, such as an eosin dye, or by brief exposure to ultraviolet or visible light. Additionally, water soluble polymers which include cinnamoyl groups which may be photochemically cross-linked may be utilized, as disclosed in Matsuda *et al.*, ASAID *Trans.*, 38:154-157 (1992).

The preferred polymerizable groups are acrylates, diacrylates, oligoacrylates, dimethacrylates, oligomethacrylates, and other biologically acceptable photopolymerizable groups. Acrylates are the most preferred active species polymerizable group.

Naturally occurring and synthetic polymers may be modified using chemical reactions available in the art and described, for example, in March, "Advanced Organic Chemistry", 4th Edition, 1992, Wiley-Interscience Publication, New York.

Polymerization is preferably initiated using photoinitiators. Useful photoinitiators are those which can be used to initiate polymerization of the macromers

without cytotoxicity and within a short time frame, minutes at most and most preferably seconds.

Numerous dyes can be used for photopolymerization. Suitable dyes are well known to those of skill in the art. Preferred dyes include erythrosin, phloxime, rose bengal, thonine, camphorquinone, ethyl eosin, eosin, methylene blue, riboflavin, 2,2-dimethyl-2-phenylacetophenone, 2-methoxy-2-phenylacetophenone, 2,2-dimethoxy-2-phenyl acetophenone, other acetophenone derivatives, and camphorquinone. Suitable cocatalysts include amines such as N-methyl diethanolamine, N,N-dimethyl benzylamine, triethanol amine, trithylamine, dibenzyl amine, N-benzylethanolamine, ÿisopropyl benzylamine. Triethanolamine is a preferred cocatalyst.

In another embodiment, the conditioned media, or alternatively particular extracellular matrix proteins elaborated into the media, are used to provide an excellent substance to coat sutures. The naturally secreted extracellular matrix provides the conditioned media with type I and type III collagens, fibronectin, terascin, glycosaminologycans, acid and basic FGF, TGF-α and TGF-β, KGF, versican, decorin and various other secreted human dermal matrix proteins. Similarly, the conditioned cell media of the invention or the extracellular matrix proteins derived from the conditioned media may be used to coat conventional implantation devices, including vascular prosthesis, in surgical approaches to correct defects in the body -- resulting in superior implantation devices. The implants should be made of biocompatible, inert materials, that replace or substitute for the defective function and made of either non-biodegradable materials or biodegradable materials. By coating implantation devices with the medium containing these extracellular proteins, the implant invites proper cellular attachments resulting in superior tissue at the implantation site. Thus, sutures, bandages, and implants coated with conditioned cell media, or proteins derived from the media, enhance the recruitment of cells, such as leukocytes and fibroblasts into the injured area and induce cell proliferation and differentiation resulting in improved wound healing.

In another embodiment of the present invention, the cells or tissues are encapsulated as described, *e.g.*, in U.S. Patent Nos. 5,874,099; 4,744,933; and 4,409,331, each of which is incorporated herein by reference in their entirety. In a preferred embodiment of the present invention, the encapsulation of genetically engineered cell and tissues of the present invention: (i) protects cells or tissues from destruction by the host's immune system; (ii) enables nutrients and oxygen to diffuse

into the capsule to sustain the cells or tissues; (iii) enables the cells or tissues to be readily retrieved; (iv) is biocompatible; and (v) does not induce the formation of scar tissue. In one embodiment, the capsule in which the cells and tissues are encased is biodegradable. In another embodiment, the capsule in which the cells or tissues are encased provides the shape for the development of new organs, see *e.g.*, U.S. Patent No. 5,770,417.

5.4. BIOREACTORS FOR THE DELIVERY OF GENE PRODUCTS LOCALLY OR SYSTEMICALLY

The engineered responsive cells and tissues of the present invention have a variety of applications including delivery of gene products locally or systematically. In another application of the present invention, the engineered responsive cells and tissues can be used for purposes of injection, transplantation or implantation *in vivo*. Cells or tissues injected, transplanted or implanted *in vivo* can be autologous or non-autologous. Furthermore, cells or tissues of the present invention can be primary or secondary cells or immortalized cell lines. In one embodiment of the present invention, the cells/tissues and the stromal cells and elements are obtained from the individual who is to receive the transplant or implant. This approach might be especially advantageous where immunological rejection of the transplant and/or graft versus host disease is likely. Moreover, fibroblasts and other stromal cells and/or elements can be derived from the same type of tissue to be cultured in the three-dimensional system. This might be advantageous when culturing tissues in which specialized stromal cells may play particular structural/functional roles, *e.g.*, glial cells of neurological tissue, Kupffer cells of liver etc.

Transfected primary or secondary cells or cell strains as described herein have wide applicability as a vehicle or delivery system for therapeutic products, such as enzymes, hormones, cytokines, antigens, antibodies, clotting factors, regulatory proteins, transcription proteins, receptors, structural proteins, novel (non-naturally occurring) proteins and nucleic acid products, such as anti-sense RNA, ribozymes, and engineered DNA. For example, transfected primary or secondary cells can be used to supply a therapeutic protein, including but not limited to, Factor VIII, Factor IX, erythropoietin, alpha-1 antitrypsin, calcitonin, glucocerebrosidase, growth hormone, low density lipoprotein (LDL), apolipoprotein E, IL-2 receptor and its antagonists, insulin, globin, immunoglobulins, catalytic antibodies, the interleukins, insulin-like growth factors, superoxide dismutase, immune responder modifiers, parathyroid hormone and interferon, nerve growth factors, tissue plasminogen activators, and colony stimulating factors.

Alternatively, transfected primary and secondary cells can be used to immunize an individual (i.e., as a vaccine).

The wide variety of therapeutic cells and tissues described herein may be engineered to deliver the following therapeutic products: a secreted protein with predominantly systemic effects; a secreted protein with predominantly local effects; a membrane protein imparting new or enhanced cellular responsiveness; membrane protein facilitating removal of a toxic product; a membrane protein marking or targeting a cell; an intracellular protein; an intracellular protein directly affecting gene expression; an intracellular protein with autolytic effects; gene product-engineered DNA which binds to or sequesters a regulatory protein; a ribozyme; and antisense-engineered RNA to inhibit gene expression.

The primary or secondary cells produced according to the present invention can be used to administer therapeutic proteins (*e.g.*, hormones, enzymes, clotting factors) which are presently administered intravenously, intra-muscularly or subcutaneously, which require patient cooperation and, often medical staff participation. In addition, transfected primary or secondary cells of the present invention produce the therapeutic product as it would normally be produced.

An advantage to the use of transfected primary or secondary cells of the present invention is that by controlling the number of cells introduced into an individual, one can control the amount of the product delivered to the body. In addition, in some cases, it is possible to remove the transfected cells or graft if there is no longer a need for the product. A further advantage of treatment by use of transfected primary or secondary cells of the present invention is that production of the therapeutic product can be regulated such as through the administration of zinc, steroids or an agent which affects translation or transcription of a protein, product or nucleic acid product or affects the stability of a nucleic acid product.

5.5. SPECIFIC EMBODIMENTS OF THE PRESENT INVENTION

In one embodiment of the present invention, the cells or tissues are engineered to express a target gene product in response to a physiological change in glucose, insulin or glucagon levels. In accordance with this embodiment, cells and tissues are genetically engineered to express a first gene product that responds to a physiological change in glucose, insulin or glucagon levels, and in response to the physiological change induces the expression of a target gene(s). The target gene(s) may correct, modify, regulate the physiological condition or indicate that medical attention is needed.

In particular embodiment, the cells of the present invention are engineered to respond to high levels of glucose and upon stimulation with glucose would express insulin. In accordance with this embodiment of the present invention, the cells and tissues may be engineered to express glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 1 derivatives (GLP-1 derivatives). GLP-1 derivatives include truncated derivatives GLP-1 (7-37), GLP-1 (7-36), GLP-1 (7-35) GLP-1 (7-34) and other truncated carboxy-terminal aminated derivatives and derivatives of GLP-1 which have amino acid substitutions, deletions, additions or other alterations (*e.g.*, addition of a non-amino acid component) which result in biological activity or stability in the blood which is substantially the same as that of a truncated GLP-1 derivative or enhanced biological activity or stability in the blood (greater than that of a truncated GLP-1 derivative). GLP-1 derivatives, also known as insulinotropins or incretins, are normally secreted into the circulation by cells in the gastrointestinal tract. *In vivo* studies have demonstrated that these peptides function to stimulate insulin secretion and inhibit glucagon secretion from the endocrine pancreas, as well as increase insulin sensitivity in peripheral tissues (Goke, 1991 Eur. J. Clin. Inv. 21:135-144; Gutniak et al., 1992 New Engl. J. Med. 326:1316-1822). Patients with non-insulin dependent diabetes mellitus (NIDDM) are often treated with high levels of insulin to compensate for their decreased insulin sensitivity. Thus, the stimulation of insulin release and the increase in insulin sensitivity by GLP-1 derivatives would be beneficial for NIDDM patients. Of particular importance is the fact that the insulinotropin-induced stimulation of insulin secretion is strongly dependent on glucose levels, suggesting that these peptides act in response to increases in blood glucose *in vivo* to potentiate insulin release and, ultimately lower blood glucose.

In another embodiment of the invention, the cells and tissues are genetically engineered to be responsive to hypoxia. In accordance with this embodiment, the cells and tissues are engineered to express a receptor that responds to hypoxia and in turn induces the expression of VEGF and/or Flt-1, which is under the control of a hypoxia-inducible enhancer element such as disclosed in, *e.g.*, Gerber et al., 1997, J. of Biological Chemistry 272:23659-23667. The expression of the VEGF and/or Flt-1 results in increased blood flow and in turn, increased levels of oxygen.

In another embodiment, cells are engineered to respond to high levels of cholesterol circulating as low density lipoprotein particles or LDLs. In such an embodiment the cells may be engineered to express the LDL receptor, which would bind apolipoproteins B and E, and in turn be induced to express and secrete apolipoprotein E or apolipoprotein Al, which function to bind cholesterol and direct it to the liver for degradation. Once the level

of cholesterol has been normalized, cells will no longer be induced to express apolipoproteins.

In another embodiment of the present invention, the cells and tissues are engineered to be responsive to a viral protein. In this embodiment, the cells or tissues may be engineered to express a cell surface receptor for the target virus, *e.g.* the CD4 or fusin receptor for HIV, and express an anti-HIV agent, *e.g.* a ribozyme targeted to HIV gag under the control of a HIV tat responsive promoter. In yet another environment, liver cells which express the receptor for Hepatitis B Virus (HBV) may be engineered to express an anti-HBV agent, *e.g.* a ribozyme targeted to one of the four conserved in RNAs which encode all HBV proteins, under the control of the HBx responsive enhancers HBEn I, which extends from nucleotide 950 to 1150 of the 3.2 kb HBV genome or HBEn II, which extends from nucleotide 1646 to 1715 of the HBV genome. (Spandau et al. 1988, J. Virol. 62: 427-434).

In another embodiment of the present invention, the therapeutic cells and tissues are engineered to be hyper-responsive to a viral protein. In this embodiment, the cells or tissues may be engineered to express a high-affinity cell surface receptor for the target virus, *e.g.* a modified form of CD4 or fusin receptor for HIV, and express an anti-HIV agent, *e.g.* a ribozyme targeted to HIV gag under the control of a HIV tat responsive promoter.

In another embodiment, responsive cells and tissues are engineered to express an antigenic polypeptide and interleukin-2 (IL-2) in response to a viral infection. For example, a cell may be engineered to express the cell surface receptor for HTLV, HBV or HIV, and additionally be engineered to express the HTLV surface antigen, the HBV surface antigen or the HIV env protein and IL-2 under the control of the viral transactivator. Thus, upon exposure to the virus, the cells and tissues secrete IL-2 which activates an immune response and viral antigens to prime the immune response.

In another embodiment of the present invention, therapeutic cells and tissues are engineered to secrete a gene product to replace a deleted or mutated gene product as a result of genetic or hereditary disease. For example, for a subject suffering from Hemophilia A, the cells and tissues would express Factor VIII; Hemophilia B, the cells and tissues would express Factor IX; Alpha thalessemia, the cells and tissues would express alpha globin; Beta thalassemia, the cells and tissues would express beta globin; Nieman-Pick disease, the cells and cells and tissues would express sphingomyelinase; Tay-Sachs disease, the cells and tissues would express lysosomal hexosaminidase.

In another embodiment, cells are engineered to secrete a gene product to augment deficient or defective gene expression. For example, Apolipoprotein E isoform deficiencies have been associated with susceptibility to Alzheimer's disease. In this case the engineered cells and tissues could be used to secrete the deficient ApoE isoform. In another example, carbonic anhydrase II deficiency is associated with a variety of genetic and acquired kidney diseases. The engineered cells and tissues could be used to secrete carbonic anhydrase II.

In another embodiment of the present invention, stromal cells, such as fibroblasts, are engineered to be indicator cells and tissues. In accordance with this aspect of the present invention, the stromal cells are transfected with nucleotides encoding the reporter gene under the control of viral regulatory elements, such as HIV tat. The stromal cells are cotransfected with nucleotides encoding a cell surface receptor for the particular virus *e.g.*, CD4 or fusin. The transfected cells are grown in culture using a three-dimensional cell and tissue culture system. The bioreactor is grafted onto the subject. Such a bioreactor would be useful in the case of a subject who had been exposed to the human immune deficiency virus (HIV) to determine when and if a viral infection would develop. The subject would apply a formulation containing the reporter gene substrate to assay for the presence of the virus -- once a positive result, as demonstrated by a change in the color of the skin graft, was determined the subject would know to seek medical attention.

In another embodiment of the invention, the cells and tissues are engineered to be responsive to a tumor antigen. This embodiment of the invention would be useful for a subject that was in a high risk group for a particular type of cancer -- or for a subject that had been previously treated for a cancer to determine if there was a recurrence of the cancer. For example, stromal cells may be engineered to express a cell surface receptor for the antigen, PMSA, which is associated with prostate cancer. The cells are also be engineered to express a reporter gene under the control of an inducible promoter which would respond to the receptor binding PMSA. These stromal cells are grown in three-dimensional culture systems to a size appropriate for a skin patch. Such a skin patch could be grafted to a patient who had been treated for prostate cancer in order for the patient to self-monitor for recurrence of the cancer. In yet another embodiment the cells are engineered to respond to breast tumor antigens, liver tumor antigens, colon cancer antigens, lung cancer antigens etc.

In yet another embodiment of the invention, the cells and tissues are genetically engineered to be responsive to controlled substances such as cocaine and heroine. In accordance with this embodiment, cells engineered to express a receptor that responds to

the presence of a substance and induces the expression of an indicator gene such as green fluorescent protein. The engineered cells are encapsulated in a skin patch which enables an individual (*e.g.*, the police) to detect substance abuse.

5.6. SCREENING ASSAYS

In one embodiment, the genetically engineered cells and tissues of the present invention are used to identify agents that modulate (*e.g.*, upregulate or downregulate) the expression of a gene directly or indirectly. In a preferred embodiment, the genetically engineered cells and tissues of the present invention can be used to identify agents that modulate the expression of a target gene. Examples of agents, candidate compounds or test compounds include, but are not limited to, nucleic acids (*e.g.*, DNA and RNA), carbohydrates, lipids, proteins, peptides, peptidomimetics, small molecules or other drugs. In accordance with these embodiments, modulators of the expression of a gene are identified by contacting the engineered cells or tissues of the present invention with a candidate compound and determining the expression of the mRNA or protein encoded by the gene. The level of expression of the mRNA or protein in the presence of the candidate compound is compared to the level of expression of the selected mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of expression of the gene product based on this comparison. For example, when expression of the mRNA or protein encoded by a gene is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of the mRNA or protein expression. Alternatively, when expression of the mRNA or protein encoded by the gene is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of the mRNA or protein expression. The level of expression of an mRNA or protein encoded by a gene can be determined by methods known to those of skill in the art. For example, mRNA expression can be assessed by Northern blot analysis or RT-PCR, and protein levels can be assessed by western blot analysis.

In another embodiment, the genetically engineered cells and tissues of the present invention are used to identify agents that modulate the activity of a gene product. In a preferred embodiment, the genetically engineered cells and tissues of the present invention are used to identify agents that modulate the activity of a target gene product. In accordance with these embodiments, modulators of the activity of a gene product are identified by contacting the engineered cells or tissues of the present invention with a test

compound or a control compound (*e.g.*, H_2O) and determining the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the gene product or a biologically active portion thereof. The activity of the gene product can be assessed by detecting induction of a cellular second messenger of the gene product (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), detecting catalytic/enzymatic activity of the target on an appropriate substrate, detecting the induction of a reporter gene (*e.g.*, a regulatory element that is responsive to a polypeptide of the invention operably linked to a nucleic acid encoding a detectable marker, *e.g.*, luciferase), or detecting a cellular response, for example, cellular differentiation, or cell proliferation. Techniques known to those of skill in the art can be used for measuring these activities (see, *e.g.*, U.S. Patent No. 5,401,639, which is incorporated herein by reference). The candidate compound can then be identified as a modulator of the activity of a gene product by comparing the affects of the candidate compound to the control compound.

In another embodiment, the genetically engineered cells and tissues of the present invention are used to identify pharmaceutical compositions that modulate the expression of a gene. In a preferred embodiment, the genetically engineered cells and tissues of the present invention are used to identify pharmaceutical compositions that modulate the expression of a target gene. In accordance with these embodiments, the genetically engineered cells and tissues of the present invention are implanted or injected in non-human animals (*e.g.*, mice, rats, monkeys, rabbits, and guinea pigs), a pharmaceutical composition or a control composition is administered to the animals, and the effect of a pharmaceutical composition on gene expression is determined. A pharmaceutical composition that alters the expression of a gene can be identified by comparing the level of selected mRNA and/or protein expression (*i.e.*, the mRNA or protein encoded by the gene) of an animal treated with a control composition to an animal treated with a pharmaceutical composition. Techniques know to those of skill in the art can be used to determine the mRNA and protein levels, for example, *in situ* hybridization. The animals may or may not be sacrificed to assay the affects of a pharmaceutical composition.

In another embodiment, the genetically engineered cells and tissues of the present invention are used to identify pharmaceutical compositions that modulate the activity of a gene product. In a preferred embodiment, the genetically engineered cells and tissues of the present invention are used to identify pharmaceutical compositions that modulate the activity of a target gene product. In accordance with these embodiments, the genetically engineered cells and tissues of the present invention are implanted or injected in non-human animals (*e.g.*, mice, rats, monkeys, rabbits, and guinea pigs), a

pharmaceutical composition or a control composition is administered to the animals, and the effect of a pharmaceutical composition on the activity of a gene product is determined. A pharmaceutical composition that alters the activity of a gene product can be identified by comparing the by animals treated with a control composition to an animal treated with a pharmaceutical composition. The activity of the gene product can be assessed by detecting induction of a cellular second messenger of the gene product (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), detecting catalytic/enzymatic activity of the target on an appropriate substrate, detecting the induction of a reporter gene (*e.g.*, a regulatory element that is responsive to a polypeptide of the invention operably linked to a nucleic acid encoding a detectable marker, *e.g.*, luciferase), or detecting a cellular response, for example, cellular differentiation, or cell proliferation. Techniques known to those of skill in the art can be utilized to detect changes in the activity of a gene product (see, *e.g.*, U.S. Patent No. 5,401,639, which is incorporated herein by reference).

6. EXAMPLE: CELLS AND TISSUES GENETICALLY ENGINEERED TO EXPRESS FACTOR IX

Three-dimensional, fibroblast cultures using cells transfected with Factor IX were used to assess the characteristics of the engineered fibroblasts as a gene delivery system. Therapeutic fibroblasts were engineered by introducing the Factor IX nucleotide sequences via transfection with retroviral vectors carrying Factor IX driven by the long terminal repeat regulatory elements. The transfected fibroblasts were grown into three-dimensional culture using both vicryl and nylon as scaffolds. These cultures secreted Factor IX into the medium at measurable levels for at least 12 days. At harvest the cultures were transplanted subcutaneously to nude mice and the blood concentrations recorded.

The engineered fibroblasts that secreted growth hormone was constructed using cells transfected with retroviral vectors carrying the rat growth hormone gene driven by the CMV promoter.

7. EXAMPLE: CELLS AND TISSUES ENGINEERED TO REDUCE INFLAMMATION AND REJECTION

A major complication of ulcers is the high concentration of neutrophil elastase to be found in them that destroys growth factors and receptors (Grinnell, F. & M. Zhu, 1996 J. Invest. Dermatol., 106 (2): 335-41). Tissue engineered products that inhibit neutriphil elatase can be devised by inducing the cells to secrete a neutrophil elastase inhibitor such as elafin (van Bergen, B.H. et al., 1996 Arch. Dermatol. Res., 288(8): 458-62. or HEI

(Sugimori, T. et al. 1995. Am. J. Respir. Cell Mol. Biol. 13(3): 314-22). Elafin is secreted by epithilial cells that are absent in chronic woulds. Tissue engineered constructs secreting these proteins may be beneficial in a variety of circumstances, including fibrosis and tumor metatasis (Taooka, Y. et al., 1997, Am. J. Respir. Crit. Care Med. 156 (1): 260-5.; Starcher, B. et al., 1996, J. Invest. Dermatrol., 107(2): 159-63). Genes for elafin or a related portease inhibitor might be transfected into a three-dimensional tissue construct on a suitable controllable or non-controlled promoter, and used to reduce tissue destruction in several diseases arising from abnormalities in tissue repair. Examples of diseases to be treated include ulcers, tumor metastasis, lung fibrosis etc.

8. EXAMPLE: SHORT TERM EXPRESSION

The best mode of application of short-term gene expression is to transfect three-dimensional cultures using adenoviral vectors carrying genes optimized for human codon usage, driven by a powerful promoter, such as CMV, collagen, actin, RSV that may or may not shut down. The cells are then grown in a three-dimensional scaffold to form a tissue. The scaffold may be degradable, slowly degradable scaffolds must be removed, but make it easier to take out the transplanted tissue, as the cells do not move away as much. Degradable scaffolds will eventually cease to contain the transplant. However, they do not need to be removed. The rate of degradation of the scaffold can be adjusted to provide the optimal relationship between ease of removal, cell localization and convenience.

9. EXAMPLE: METHODS FOR ALTERING DIFFERENTIATION

The differentiation of cells depends on specific patterns of gene expression. These patterns are in many cases induced through growth factor and cytokine responses. However, in every case, the surface reactions must be translated through asignal transduction pathway to the nucleus. Thus cellular differentiation might be controlled either through external proteins or through intracellular factors. Transfection provides a means to bring about the controlled expression of a secreted or intracellular protein. In general, DNA molecules are easier to prepare and purify than proteins. In order to induce a specific pattern of differentiation, a culture might be transfected so as to cause the transient expression of a secreted growth factor that would then induce the appropriate response, or directly by inducing synthesis of an intracellular switching protein, such as a master gene.

10. EXAMPLE: METHODS FOR SELECTING SLOWLY CYCLING CELLS

Slowly cycling cells are of interest because this population includes stem cells. Slowly cycling cells might be enriched by transfecting cells with herpes simplex virus thymidine kinase, using a viral vector such as a retroviral vector that is unable to integrate in non-dividing cells. Cells in which the vector has been able to integrate are destroyed with gangcyclovir, thus enriching the population for stem cells. This technique might be combined with other stem cell selection methods.

11. EXAMPLE: METHODS FOR REVERSIBLE IMMORTALIZATION

Certain cell types, of which primary fibroblasts are an example, have a limited lifespan. It is, therefore, very difficult to clone them or select for cells showing desirable culture or gene expression characteristics. Immortalization can be achieved using telomerase. However, immortal cells may have considerable disadvantages in therapeutic applications. A way to avoid this is to insert the telomerase gene between *loxP* sites that can be activated to recombine, and thus excise the telomerase gene, by induction of *cre*. In combination with various backup safeguards, such as including the herpes simplex virus thymidine kinase within the *loxP* sites or an antibiotic resistance gene separated from its promoter by the *loxP* delimited insert, this method can provide reliable reversible immortality on the cells. Such a technique could be applied to cells on a scaffold.

12. EXAMPLE: METHODS FOR REGULATING THE EXPRESSION OF A TARGET GENE

Stromal cells co-transfected with pCI-CD4 and pHIVtat-ribozyme are grown in a three-dimensional cell culture system. The pCI-CD4 construct consists of the human CD4 cloned into the pCI mammalian expression vector (Promega Corporation, Madison, Wiscosin), and the pHIVtat-ribozyme construct consists of an HIV-ribozyme (see, *e.g.*, Wong-Staal, F. et al., 1998, Hum Gene Ther 9(16):2407-25) cloned into an HIV-tat inducible expression vector (see, *e.g.*, Frasier, C. et al., 1998, Gene Ther 12:1665-76). The stromal cells are encased in a biocompatible capsule such as described in U.S. Patent No. 5,874,099 and implanted *in vivo*. HIV binds to the constitutively expressed CD4 molecule on the encapsulated stromal cells, infects the cells, and the expression of the HIV tat protein induces the expression of the HIV-ribozyme, which cleaves HIV RNA. The

advantage of this system is that HIV-ribozymes will produced in response to the presence of the virus.

Alternatively, stromal cells can be co-transfected with pCI-CD4 and pHIVtat-GRP, grown in a three-dimensional cell culture system, and encapsulated. The encapsulated cells are implanted *in vivo* in a position such that capsule is readily accessible and retrievable. The expression of the CD4 receptor allows the stromal cells to become infected with HIV, and the expression of HIV tat protein induces the expression of green fluorescent protein ("GFP"), which is by detectable fluorescence microscopy. This system enables physicians to monitor individuals that have been exposed to HIV in order to determine infection, and enables physicians to determine the best therapeutic treatment regimen.

13. EXAMPLE: SKIN PATCHES CONTAINING CELLS RESPONSIVE TO THYROID HORMONE

Stromal cells are co-transfected with pCI-TR and pTRE-GRP by techniques known to those of skill in the art. The pCI-TR construct consists of the nucleotide sequence encoding human thyroid hormone receptor ("TR") cloned into the pCI mammalian expression vector (Promega Corporation, Madison, Wiscosin), and the pTRE-lacZ construct consists of the nucleotide sequence encoding lacZ cloned into an expression vector. LacZ expression is under the control of the thyroid hormone response element, which has been engineered to be sensitive to high concentrations of thyroid hormone. Stromal cells expressing the genes are selected for and the stably transfected cells are encapsulated in a biocompatible patch that adheres to the skin. The constitutive expression of the thyroid receptor enables the cells to respond to thyroid hormones (*e.g.*, T4 and T3) and, in response, induce the expression of lacZ. The expression of lacZ is detectable by applying X-gal or something analogous and assessing the color of the patch. If the patch turns blue, then the thyroid hormone concentrations are high and medical attention is required. This system enables the recipient or his/her physician to monitor the thyroid hormone concentrations in individuals with thyroid diseases and/or disorders including, but not limited to, hyperthyroidism (*e.g.*, Grave's disease) and hypothyroidism conditions (*e.g.*, Hashimoto's thyroiditis).

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. An injectable or implantantable composition suitable for use in a human subject comprising a cell genetically engineered to contain at least two genes each under the control of separate regulatory elements, so that the first gene product is expressed and enables the cell to respond to an environmental cue or stimulus that regulates the expression of the second or target gene product.

2. The composition of Claim 1 in which the expression of the first gene product is constitutive.

3. The composition of Claim 1 in which the first gene product is a receptor.

4. The composition of Claim 1 or 3 in which the first gene product is overexpressed.

5. The composition of Claim 3 in which the receptor is a chimeric receptor.

6. The composition of Claim 1 in which the target gene is a reporter gene.

7. The composition of Claim 6 in which the expression of the target gene indicates the need for medical attention.

8. The composition of Claim 1 in which the target gene corrects, modifies or regulates the physiological condition to which the engineered cell responds.

9. The composition of Claim 1 in which the expression of the target gene has anti-viral, anti-bacterial, anti-microbial, or anti-cancer effect.

10. The composition of Claim 1 in which the environmental stimulus or cue is a physiological change in glucose, insulin or glucagon levels.

11. The composition of Claim 1 in which the target gene is a growth factor, hormone or cytokine.

12. The composition of Claim 1 further engineered to express a gene that results in the immortalization of said cell.

13. The composition of Claim 12 in which the cell is reversibly immortalized.

14. The implantable composition of Claim 1 in which the genetically engineered cells are grown on a living stromal tissue prepared *in vitro* comprising stromal cells and connective tissue proteins naturally secreted by the stromal cells attached to and substantially enveloping a framework composed of a biocompatible non-living material formed into a three-dimensional structure having interstitial spaces bridged by the stromal cells.

15. The implantable composition of Claim 14 in which the genetically engineered cells are stromal cells.

16. The implantable composition of Claim 14 in which the genetically engineered cells are parenchymal cells.

17. The injectable composition of Claim 1 comprising the genetically engineered cells and a polymerizable or cross-linking hydrogel.

18. The injectable composition of Claim 17 in which the hydrogel is an aliginate.

19. The injectable composition of Claim 17 in which the hydrogel is photopolymerizable.

20. The injectable composition of Claim 1 comprising the genetically engineered cells and a carrier composed of extracellular matrix proteins.

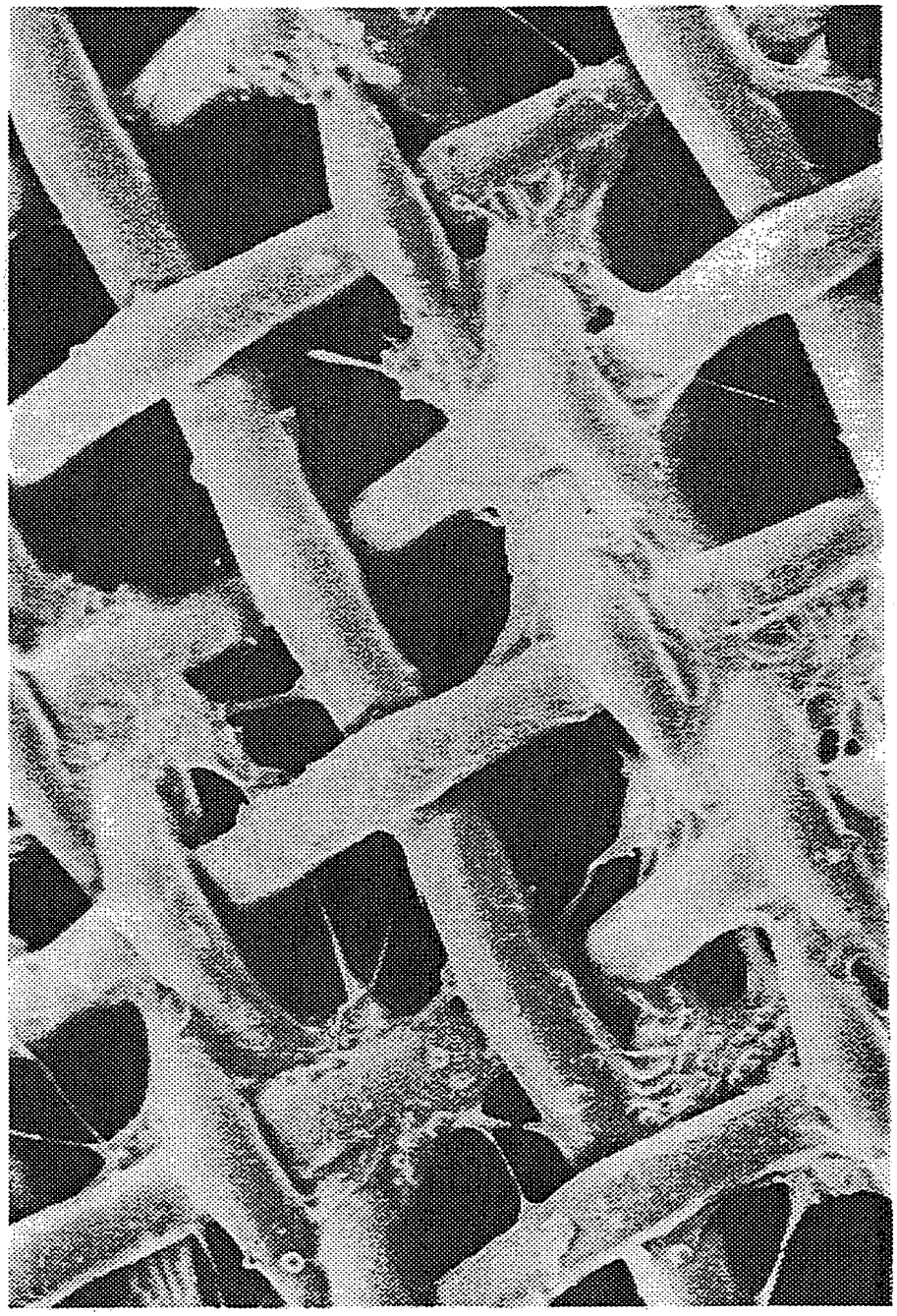

FIG. 1

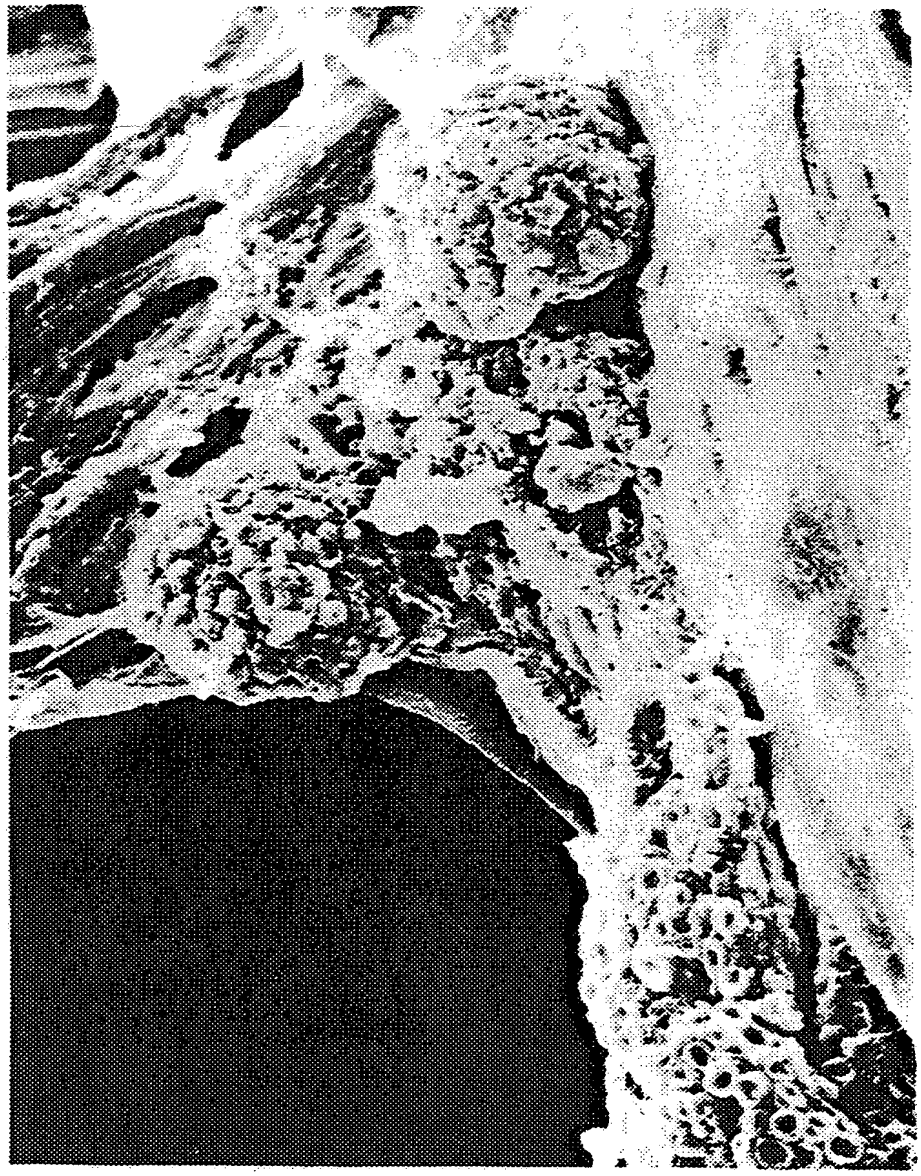

FIG.2

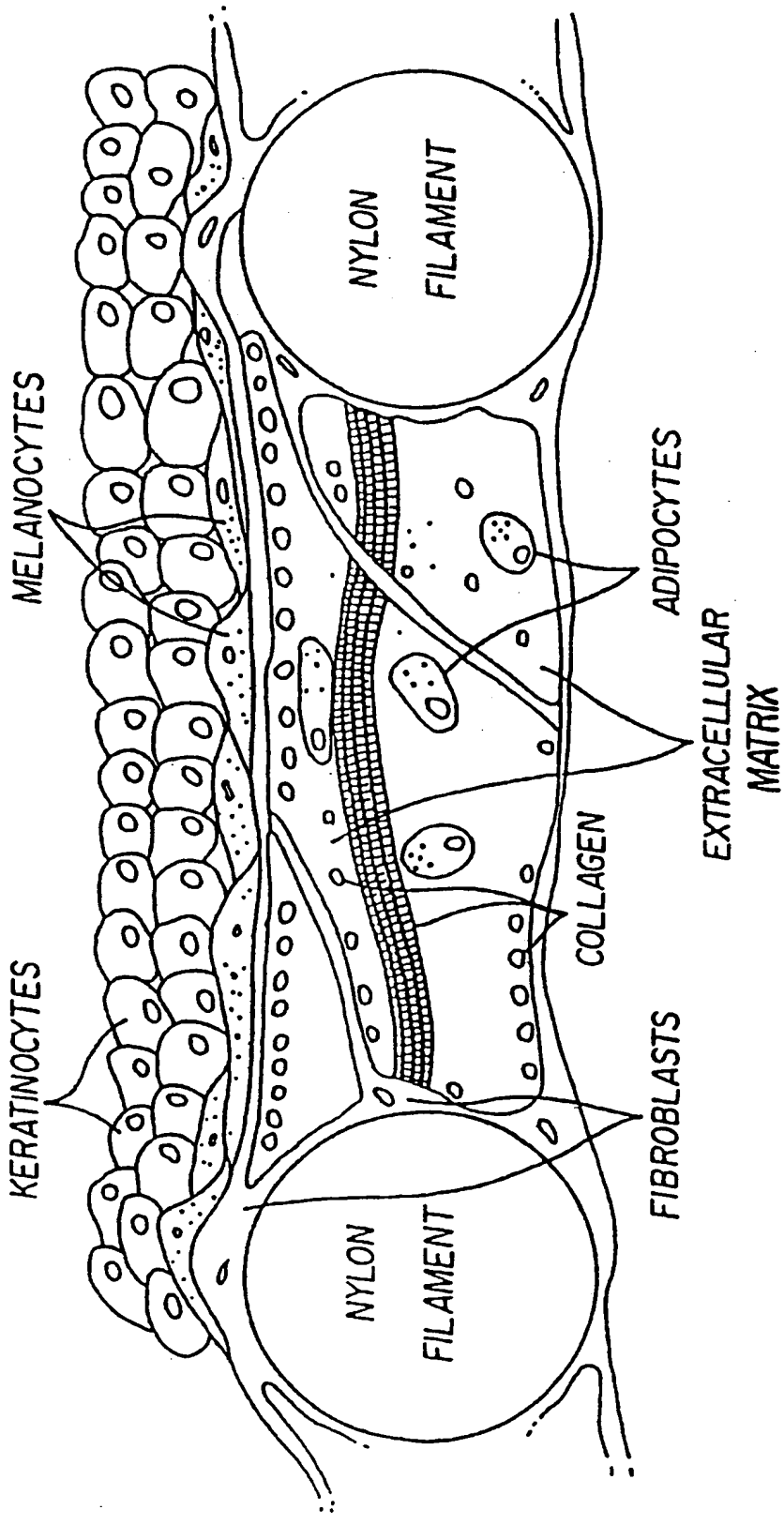
NYLON FILAMENT
MELANOCYTES
ADIPOCYTES
EXTRACELLULAR MATRIX
COLLAGEN
KERATINOCYTES
FIBROBLASTS
NYLON FILAMENT

FIG.3

FIG.4

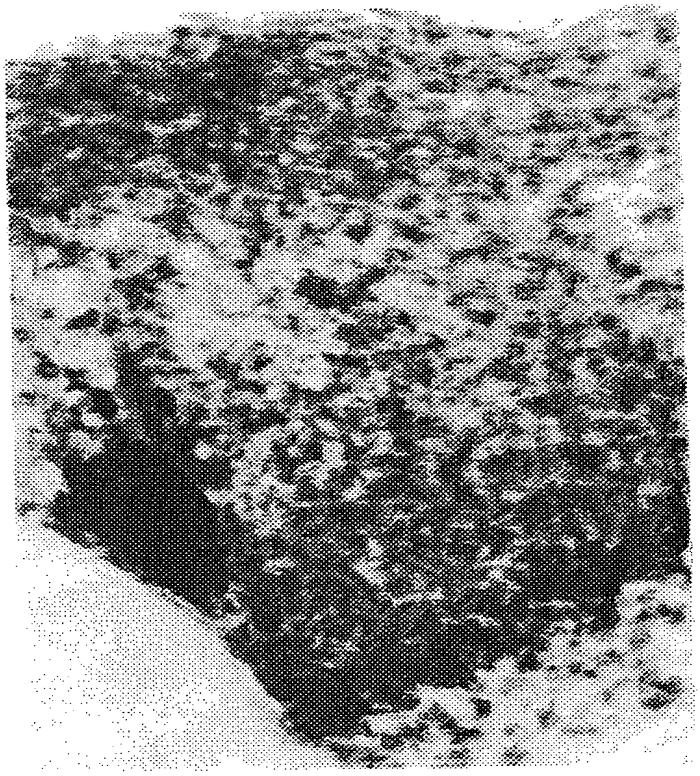

FIG.5

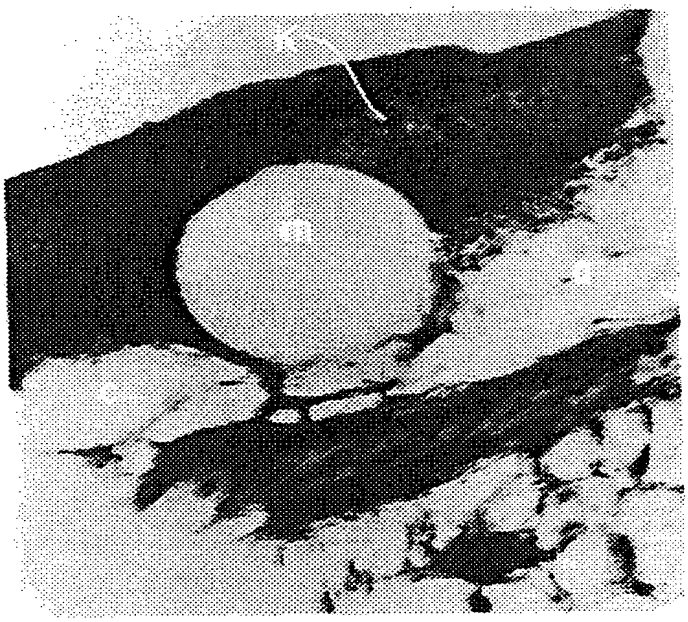

FIG.6

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 48/00, C12N 5/10

A3

(11) International Publication Number: WO 00/06204

(43) International Publication Date: 10 February 2000 (10.02.00)

(21) International Application Number: PCT/US99/16961

(22) International Filing Date: 28 July 1999 (28.07.99)

(30) Priority Data:
60/094,408 28 July 1998 (28.07.98) US

(71) Applicant: ADVANCED TISSUE SCIENCES, INC. [US/US]; 10933 North Torrey Pines Road, La Jolla, CA 92037-1005 (US).

(72) Inventors: NAUGHTON, Gail, K.; 10933 North Torrey Pines Road, La Jolla, CA 92037-1005 (US). MANSBRIDGE, Jonathon, N.; 1685 Calle Camille, La Jolla, CA 92037 (US).

(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW. ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
4 May 2000 (04.05.00)

(54) Title: GENETICALLY ENGINEERED CELLS AND TISSUES

(57) Abstract

The present invention relates to cells genetically engineered to express a target gene in response to a given "environmental cue" or "stimulus", and their use for the production of three-dimensional tissues or injectable preparations which can be used in tissue repair, replacement or enhancement, and/or for the delivery of therapeutic gene products *in vivo*. In particular, the invention relates to cell and tissue bioreactors that are engineered to express a target gene product, which acts as a reporter of a chosen physiological condition, augments deficient or defective expression of a gene product or provides an anti-viral, anti-bacterial, anti-microbial, or anti-cancer activity in response to a given "environmental cue" or "stimulus".

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/16961

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K48/00 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 40175 A (ADVANCED TISSUE SCIENCES, INC.) 19 December 1996 (1996-12-19) page 7, line 10 -page 8, line 32 page 32, line 5 -page 44, line 14	1-20
A	WO 98 20041 A (OKLAHOMA MEDICAL RESEARCH FOUNDATION) 14 May 1998 (1998-05-14) page 7, line 3 - line 21; claims page 2, line 7 -page 3, line 11	1-20
A	WO 95 21927 A (ISIS INNOVATION LIMITED) 17 August 1995 (1995-08-17) page 2, line 23 -page 3, line 8; claims	1-20

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 February 2000	15/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In. itional Application No
PCT/US 99/16961

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 99 62562 A (UNIVERSITY OF WASHINGTON) 9 December 1999 (1999-12-09) page 33, line 1 -page 34, line 14 page 39, line 1 - line 15; claims 9,15-18,27,33-36	1,2,10, 11

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/16961

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9640175 A	19-12-1996	US 5863531 A	26-01-1999
		AU 706426 B	17-06-1999
		AU 6031596 A	30-12-1996
		CA 2224071 A	19-12-1996
		EP 0831861 A	01-04-1998
		JP 11506611 T	15-06-1999
		NZ 310004 A	28-10-1999
WO 9820041 A	14-05-1998	AU 5431798 A	29-05-1998
		EP 0937104 A	25-08-1999
WO 9521927 A	17-08-1995	EP 0745131 A	04-12-1996
		JP 9508531 T	02-09-1997
		US 5942434 A	24-08-1999
WO 9962562 A	09-12-1999	NONE	